The phosphatidylinositol signal transduction system in the yeast Saccharomyces cerevisiae

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THE PHOSPHATIDYLINOSITOL SIGNAL TRANSDUCTION SYSTEM

IN THE YEAST *SACCHAROMYCES CEREVISIAE*

Submitted by Kevin Spencer Robinson

for the degree of Ph. D.

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Abstract

The phosphatidylinositol (PI) signal transduction system, involving agonist-stimulated hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce the secondary messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, has been well established in mammalian cells. The primary aim of this project was to determine whether a PI transduction system existed in the yeast *Saccharomyces cerevisiae*, and if it did, to determine to which "agonist" it was responsive.

Based on the work of Kaibuchi *et al.* (1986), who reported increased IP_3 production in response to glucose stimulation, research was based around three objectives: to detect the presence, synthesis and degradation of IP_3.

It was not possible to confirm the findings of Kaibuchi *et al.* (1986), but the same methodology was used to demonstrate the breakdown of radiolabelled inositol phospholipids to their deacylated products in response to glucose-stimulation, presumably as a function of phospholipase A activity. IP_3 was not isolated from yeast throughout the project but it was possible to extract materials that co-eluted with known standards of IP_2, IP_1 and GPI. The uptake of tritiated inositol was demonstrated. Extracted lipids were then separated and radioactivity was shown to be incorporated into the membrane phosphoinositides. Evidence is provided to demonstrate the presence of an inositol

phosphate degradation pathway, exogenous ^{3}H-IP_3 being sequentially dephosphorylated to IP_2, IP_1 and inositol. IP_3 kinase activity was also demonstrated, with the production of IP_4 from ^{3}H-IP_3. The attempted conversion of exogenous PIP_2 to IP_3 using a yeast cell preparation was unsuccessful and no turnover was seen.

If the PI system has ancient origins, it may be present in simple eukaryotes albeit with different functions. Results suggested the presence of a transduction system, but one in which IP_3 did not necessarily play a major role.

Acknowledgements

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Contents

1.0 Introduction

1.1 Cell to Cell Signalling

No cell lives in isolation (Darnell *et al.*, 1986). The evolution of multicellular organisms has depended on the ability of cells to communicate with each other. Communication between cells is required to regulate their development and organization into tissues, to control their growth and division, and to coordinate their diverse activities (Alberts *et al.*, 1983).

Cells are thought to communicate in 3 different ways: (1) they secrete chemicals that signal to cells some distance away; (2) they display plasma-membrane-bound signalling molecules that influence those cells that make direct physical contact; and (3) they form gap junctions that join the cytoplasms of interacting cells.

Some signals induce a modification in the activity of one or more enzymes already present in the target cell. This type of reaction allows the cell to respond quickly. Most signalling molecules that induce such rapid changes are water soluble and bind to receptors located in the plasma membrane. Other signalling molecules primarily alter the pattern of gene expression. These molecules are generally lipid soluble and induce slower, longer lasting responses.

The ability of a cell to respond to a particular extracellular signalling molecule depends on its having

specific proteins, *viz* receptors, that bind the signalling molecule. Cell surface receptors act as "molecular antennae" (Berridge and Irvine, 1984) and scan the environment for carriers of information such as hormones, growth factors and neurotransmitters which are transmitted across the cell membrane (Majerus *et al.*, 1986) to be transduced and amplified into internal signals. The response of a cell to a group of hormones is dictated both by the set of receptors it possesses and by the intracellular reactions initiated by the binding of any one hormone to its receptors (Darnell *et al.*, 1986).

The great majority of cell-surface receptors that bind hydrophilic signalling molecules are thought to undergo a conformational change when they bind to a ligand at the cell exterior. This change leads to the generation of an intracellular signal that alters the behaviour of the target cell. The intracellular signalling molecule is referred to as a second messenger, the first messenger being the extracellular ligand itself (Berridge, 1987a, 1989; Hokin, 1985).

Cell-surface receptors are known to generate intracellular signals in one of two ways. Some systems involve the activation or inactivation of a plasma-membrane bound enzyme. The enzyme may then catalyse the production of an intracellular mediator which acts as a second messenger simply as a result of its increase in concentration e.g. adenylate cyclase and cyclic AMP, or by phosphorylating

cellular proteins e.g. epidermal growth factor. Other systems open or close gated ion channels in the plasma membrane. The generated signal can either alter the voltage across the plasma membrane e.g. neurotransmitters, or cause a large influx of ions into the cytosol, so initiating a cellular response e.g. calcium channels.

In most receptor-ligand systems, the ligand appears to have no function other than binding to the receptor. The ligand is not metabolized (although it may be degraded by the target cell), is not an active intermediate in cellular activity and has no enzymic properties. Its sole function is to modify the characteristics of the receptor and alert it to the presence of a specific product in the environment (Darnell *et al.*, 1986; Alberts *et al.*, 1983).

1.2 Signal Transduction and the Inositol Phospholipids

The notion that eukaryote cell behaviour is governed by signalling systems which transduce externally arriving information into a small number of secondary messengers (Berridge and Irvine, 1984) has been accepted since the 1950's. In this decade the most well known second messenger, cyclic adenosine monophosphate (cAMP) was discovered and was found to be synthesized by adenylate cyclase in response to extracellular stimulation. It was later revealed that adenylate cyclase was not directly activated, but required a GTP binding protein in the cell membrane and thus the basis of transmembrane signalling systems was established.

Inositol lipids are anionic membrane phospholipids that contain the cyclic polyalcohol myo-inositol in their head groups, attached *via* the hydroxyl on the 1 position to a phosphate (Berridge, 1981a, 1984; Hawthorne, 1964; Hawthorne and White, 1975) (Fig. 1.1). The metabolism of these phospholipids was found to be affected by hormones (Hokin and Hokin, 1953, 1955). The muscarinic cholinergic receptor agonist, acetylcholine, selectively increased the incorporation of $^{32}P_i$ into two minor plasma membrane phospholipids of the pancreas (Hughes *et al.*, 1990; Hughes and Putney, 1990). However, it was not until 1975 that Michell (1975) suggested that receptor-stimulated inositol lipid hydrolysis somehow couples the activation of receptors to an increase in cytosolic calcium ion (Ca^{2+}) concentration. The stimulated metabolism was associated with receptor function and those agonists that raise intracellular calcium (Michell, 1975; Durrell *et al.*, 1969) and it later became clear that a stimulated catabolism of inositol-lipids occurs in many different cells in response to a wide range of external signals (Downes and Michell, 1982; Berridge, 1984; Berridge and Irvine, 1984; Hughes *et al.*, 1990; Majerus, 1992)

Mammalian cells contain three inositol phospholipids - phosphatidylinositol (PI); phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2), commonly known collectively as the phosphoinositides (Fig. 1.1). These phospholipids undergo rapid interconversions catalysed by specific kinases and phosphomonoesterases

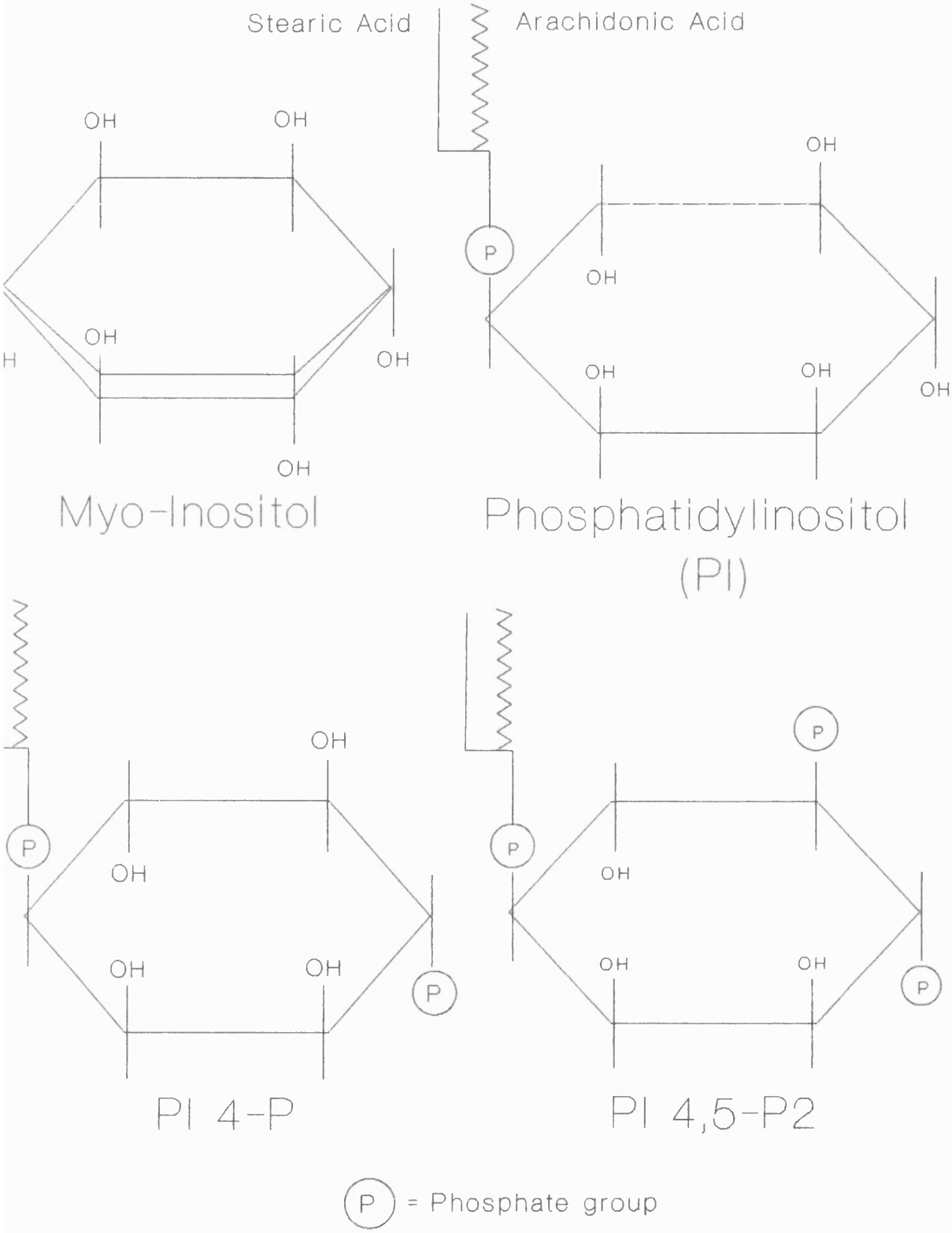


Fig. 1.1. Myo-Inositol and the Phosphoinositides

PI 4-P, Phosphatidylinositol 4-Phosphate

PI 4,5-P_2, Phosphatidylinositol 4,5-Bisphosphate

(Downes *et al.*, 1989). PIP_2, a quantitatively minor lipid confined mainly to the inner leaflet of the plasma membrane (Berridge, 1987a) is formed by the 2-stage phosphorylation of PI. PI kinase phosphorylates the hydroxyl on the 4 position of the inositol head group with an adenosine triphosphate (ATP) derived phosphate group to form PIP. This is further phosphorylated at the 5 position by PIP kinase to give PIP_2 (Hawthorne and Pickard, 1979; Downes and Michell, 1982). The conversion of PI to the polyphosphoinositides can be reversed by two phosphomonoesterases that specifically remove phosphates from the 4- and 5- positions. The distribution of the phosphate groups in the inositol rings of PIP and PIP_2 was established by Grado and Ballou (1961) and Tomlinson and Ballou (1961).

Hormones and neurotransmitters that use calcium as a second messenger specifically hydrolyse membrane phosphoinositides (Berridge, 1984) and a vast array of information has been collected documenting the agonists that act in many different cell types to augment inositide metabolism, causing the depletion of membrane PI (Michell, 1975, 1979; Berridge, 1980, 1981b, 1983; Michell and Kirk, 1981; Michell *et al.*, 1981; Putney, 1981; Irvine *et al.*, 1982).

Substantial evidence was also acquired to show that many of the same agonists could also stimulate the disappearance of the polyphosphoinositides represented by PIP and PIP_2 (Durrell *et al.*, 1968; Abdel-latif *et al.*, 1977; Akhtar and Abdel-latif, 1980; Kirk *et al.*, 1981a; Downes and Michell,

1982; Berridge, 1983). Such a perturbation of membrane phospholipids was thought to represent a fundamental transduction mechanism that resulted in the mobilization of calcium, the activation of protein kinase C, the release of arachidonic acid and stimulation of guanylate cyclase to form cyclic guanosine monophosphate (cGMP) (Berridge, 1984). A characteristic feature of this signal transduction system is the receptor mediated hydrolysis of phosphoinositides to give two products - *sn*-1,2 diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Durrell *et al.*, 1969; Abdel-latif *et al.*, 1977; Akhtar and Abdel-latif, 1980; Berridge, 1984, 1987a, 1989; Berridge and Irvine, 1989; Hughes and Putney, 1990). Berridge (1987b) states that the fact that IP_3 is produced following receptor stimulation provides unequivocal evidence that PIP_2 is the lipid used in the receptor mechanism. This is explained below.

1.3 Components of Signal Transduction

The transduction unit within the plasma membrane consists of three main components: (1) a receptor that detects the incoming signal; (2) a G-protein that serves to couple the receptor to the third component, and (3) the phosphodiesterase responsible for cleaving the lipid precursor (Berridge, 1987a). PIP_2 is hydrolysed by a specific phosphodiesterase 'phospholipase C' in the presence of a G-protein (Majerus *et al.*, 1986, 1988; Berridge and Irvine, 1984) (Fig. 1.2) to produce DAG and IP_3 which both act as second messengers.

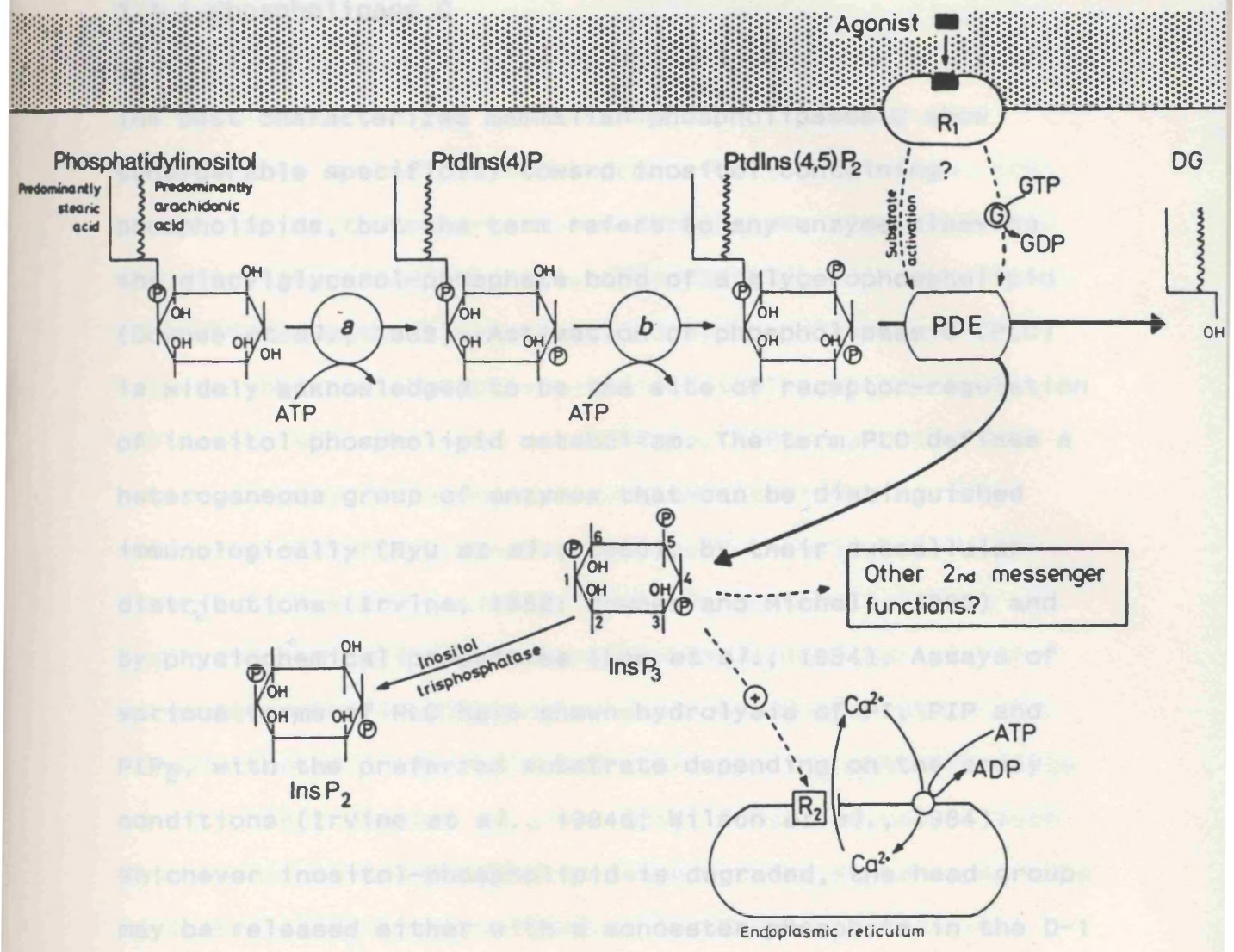


Fig. 1.2. The Proposed Role of Inositol Trisphosphate as an Intracellular Second Messenger

Agonists bind to external receptors (R_1) to stimulate the hydrolysis of PIP_2 to form diacylglycerol (DAG) and inositol trisphosphate (IP_3).

The latter may have a number of second messenger functions, one of which is to bind to a specific receptor (R_2) on the endoplasmic reticulum to release calcium. The action of IP_3 is curtailed by an inositol trisphosphatase which removes a phosphate from the 5-position to form IP_2.

a, Phosphatidylinositol kinase
b, PIP kinase
PDE Phosphodiesterase (Phospholipase C)

1.3.1 Phospholipase C

The best characterized mammalian phospholipases C show considerable specificity toward inositol-containing phospholipids, but the term refers to any enzyme cleaving the diacylglycerol-phosphate bond of a glycerophospholipid (Downes *et al.*, 1989). Activation of phospholipase C (PLC) is widely acknowledged to be the site of receptor-regulation of inositol phospholipid metabolism. The term PLC defines a heterogeneous group of enzymes that can be distinguished immunologically (Ryu *et al.*, 1986), by their subcellular distributions (Irvine, 1982; Downes and Michell, 1985) and by physiochemical properties (Low *et al.*, 1984). Assays of various forms of PLC have shown hydrolysis of PI, PIP and PIP_2, with the preferred substrate depending on the assay conditions (Irvine *et al.*, 1984a; Wilson *et al.*, 1984). Whichever inositol-phospholipid is degraded, the head group may be released either with a monoester phosphate in the D-1 position or as the corresponding 1:2 cyclic phosphate ester (Dawson and Clarke, 1972). Durrell *et al.* (1969) first provided evidence for PLC activation by demonstrating accumulation of inositol phosphates in cholinergically-stimulated synaptosomes that had been labelled with ^3H-inositol, but early experiments could not determine between direct hydrolysis of all three phosphoinositides, specific hydrolysis of one phosphoinositide with the others being consumed during resynthesis or simultaneous hydrolysis of PIP_2 and PIP with PI consumed during their resynthesis (Michell *et al.*, 1981; Abdel-latif, 1983; Downes and

Michell, 1982).

The mode of action was not elucidated until simple and reproducible methods were developed for the detection and separation of the water-soluble phosphates released by PLC activity (Downes and Michell, 1981; Berridge *et al.*, 1982, 1983). These methods were enhanced by the discovery that lithium inhibits the activity of inositol-1-phosphatase (Allison, 1978; Hallcher and Sherman, 1980), and could penetrate many different cell systems (Berridge *et al.*, 1982), so preventing the hydrolysis of Ins(1)P_1. By inhibiting the conversion of Ins(1)P_1 to inositol (and Ins(1,4)P_2 to IP_1) lithium greatly amplifies the accumulation of inositol phosphates during agonist stimulation of tissues both *in vitro* and *in vivo* (Berridge *et al.*, 1982; Sherman *et al.*, 1981, 1985). The use of such techniques provided direct evidence for the rapid appearance of Ins(1,4,5)P_3 in stimulated cells (Berridge, 1984; Berridge and Irvine, 1984; Burgess *et al.*, 1985) and provided the experimental basis for pinpointing the substrate specificity of receptor-sensitive PLC.

Subsequent investigations involving radioactive labelling of the phosphoinositides showed that PIP_2 breakdown occurs rapidly, and is usually detectable within 5 seconds of the initiation of hormonal stimulation (Creba *et al.*, 1983; Weiss *et al.*, 1982; Downes and Michell, 1985). In general, within 1 minute, the PIP_2 concentration has reached a new steady-state, between 50% and 80% of the control value. The

level of the steady-state is presumed to reflect the relative activities of the hormone-sensitive PLC and the enzymes that determine the PIP_2 concentration in an unstimulated cell. The rapid breakdown of PIP_2 is usually, but not always, accompanied by the disappearance of PIP (Creba *et al.*, 1983), however no detectable fall in cellular PI content is generally observed until a few minutes of exposure to the hormone have elapsed (Kirk *et al.*, 1981b; Martin, 1983).

An alternative theory proposed by Majerus *et al.* (1985) suggested that PIP_2 may be hydrolysed initially, but prolonged stimulation may involve PI hydrolysis too. This would allow for the production of 1,2-DAG without the simultaneous production of IP_3. Evidence to support this theory was provided by Griendling *et al.* (1986), but considering DAG metabolism and IP_3 metabolism proceed by independent pathways, it may not be unusual to see DAG accumulation without IP_3 depending on their respective rates of formation and degradation.

1.3.2 The G-Protein

A family of G-proteins or 'GTP-binding' proteins on the inner face of the plasma membrane are responsible for transducing signals across cell membranes (Neer and Clapham, 1988; Berridge, 1987b, 1987c). The classic example being the stimulatory G-protein (G_s) which couples receptors to adenylate cyclase. The G-protein involved in inositol lipid

transduction seems to differ from the above and has been abbreviated to G_p, with the subscript p referring to phospholipid (Cockroft and Gomperts, 1985). In an unstimulated state G_p is probably bound to GDP, but once a receptor is occupied it undergoes a conformational change which is transmitted to G_p, causing a loss of GDP and an enhanced affinity for GTP acting from within the cell. The arrival of GTP initiates the "on-reaction", reducing the affinity of the receptor for the ligand while simultaneously activating the PLC to hydrolyse PIP_2. The off reaction occurs when the transduction process is terminated by the hydrolysis of GTP to GDP by the GTPase activity associated with G_p (Berridge, 1987c). G_p has not been identified and its relationship to other G-proteins is not known. Wakelam *et al.* (1986) proposed that G_p may resemble or be the product of the *ras* oncogene. When NIH 3T3 cells were transformed with the N-*ras* proto-oncogene, it appeared that G_p was limiting for inositol phosphate production under normal conditions. The expression of *ras* however, increased the ability of receptors to activate the phosphoinositidase. It is unlikely though that the G_p protein that couples receptors to phospholipase C is encoded by a *ras* proto-oncogene, because then G_p would be very different from the many other G-proteins which are homologous heterotrimers; the *ras* proteins are single polypeptides and probably have an unrelated but novel function (Alberts *et al.*, 1983; Bourne and Stryer, 1992; Lefkowitz, 1992). The evidence that G_p functions in signal transduction is based primarily on the observation that the breakdown of PIP_2 to IP_3 and DAG

can be stimulated by the addition of non-hydrolyzable guanine nucleotide analogues to either permeabilized cells or to isolated membranes. G_p was also shown to be fluoride activated, a common factor in G-proteins. Fluoride ions inhibit the GTP-ase component and leave G-proteins in their active conformation. G_p reacted in the same way as other known G-proteins (Blackmore *et al.*, 1985; Strand and Wong, 1985).

1.4 Second Messenger Metabolism

A key feature in the inositol lipid signalling system is that both products of PIP_2 hydrolysis function as second messengers, thus forming a bifurcating signal pathway for transferring information into the cell (Berridge, 1987a). Water soluble $Ins(1,4,5)P_3$ is released into the cytosol and mobilizes calcium from the microsomes of the endoplasmic reticulum (ER) (Berridge, 1983, 1987a, 1989; Berridge and Irvine, 1984, 1989; Streb *et al.*, 1983). Calcium is constantly cycling across the ER membrane with IP_3 acting to stimulate the passive efflux component without altering the calcium pump. It is likely that IP_3 opens a calcium channel when bound to a putative receptor (Berridge, 1987b). The neutral DAG remains in the plane of the plasma membrane and stimulates protein phosphorylation *via* the action of protein kinase C (Nishizuka, 1983, 1984a, 1984b, 1988; Takai *et al.*, 1982). Protein kinase C (PKC) requires the presence of DAG, Ca^{2+} and phospholipid for activation (Kishimoto *et al.*, 1980). DAG is thought to increase the affinity of PKC for

calcium released into the cytosol, and transported *via* the calcium binding protein calmodulin. Nishizuka (1986) showed that DAG decreases the Ca^{2+} requirements for PKC *in vitro* to levels found in cytosols of resting cells, suggesting that an increased calcium concentration was not required. However *in vitro* studies may not represent the behaviour *in vivo*, and activation of certain PKC isoenzymes may require different calcium levels. In mammalian cells, PKC activities represent several discrete isoenzymes - α, β_I, β_{II} and Γ were isolated by screening DNA libraries and three others (δ, ϵ and ?) were found in rat brain cells (Simon *et al.*, 1991; Nishizuka, 1988). Experimentally PKC can also be activated by derivatives of DAG, phorbol esters and in some cells, the entry of calcium through voltage-dependent channels, but when both limbs of the signal pathway are activated DAG and Ca^{2+} probably act synergistically to activate the enzyme (Berridge, 1987a).

The hallmark of this inositol lipid signalling system is that there is a bifurcation in the flow of information - one limb of the pathway depends on DAG activating PKC to phosphorylate specific cellular proteins, whereas the other pathway depends on calcium which acts through calmodulin to phosphorylate a second group of proteins. The bifurcation provides the versatility necessary to introduce subtle variations in the control mechanism. The dual signal hypothesis concerns the way in which IP_3/Ca^{2+} and DAG/PKC pathways may contribute to the final response by acting either co-operatively or synergistically. The relative

importance of each pathway may also vary with time, the IP_3/Ca^{2+} pathway plays a major and direct role in initiating cellular responses whereas the DAG/PKC pathway predominantly modulates either the calcium-signalling pathway or other signal pathways, but may also contribute to the final response. It is also possible that some agonists may be able to stimulate one limb of the signal pathway but not affect the other.

Ultimately, it is essential to resynthesize the lipid precursor (PIP_2) so that the turnover cycle may continue. The two products formed during PIP_2 hydrolysis are mostly conserved by being fed into a lipid cycle and an inositol phosphate cycle that finally combine to reform PI (Berridge, 1984) (Fig. 1.3). The metabolic fates of the second messengers are described below.

1.4.1 Inositol (1,4,5) Trisphosphate

IP_3 can be metabolized by two distinct routes (e.g. Hawkins *et al.*, 1986): by dephosphorylation to form IP_2 (Downes *et al.*, 1982) or by further phosphorylation to IP_4. IP_3 is dephosphorylated by a 5-phosphate-specific phosphatase to produce $Ins(1,4)P_2$. Depending on the tissue, the proportion of intracellular 5-phosphatase activity that is membrane bound varies from over 80% down to 16% (Shears, 1989), but appears to be largely confined to the plasma membrane. $Ins(1,4)P_2$ is weak or inactive at mobilizing Ca^{2+} so the 5-

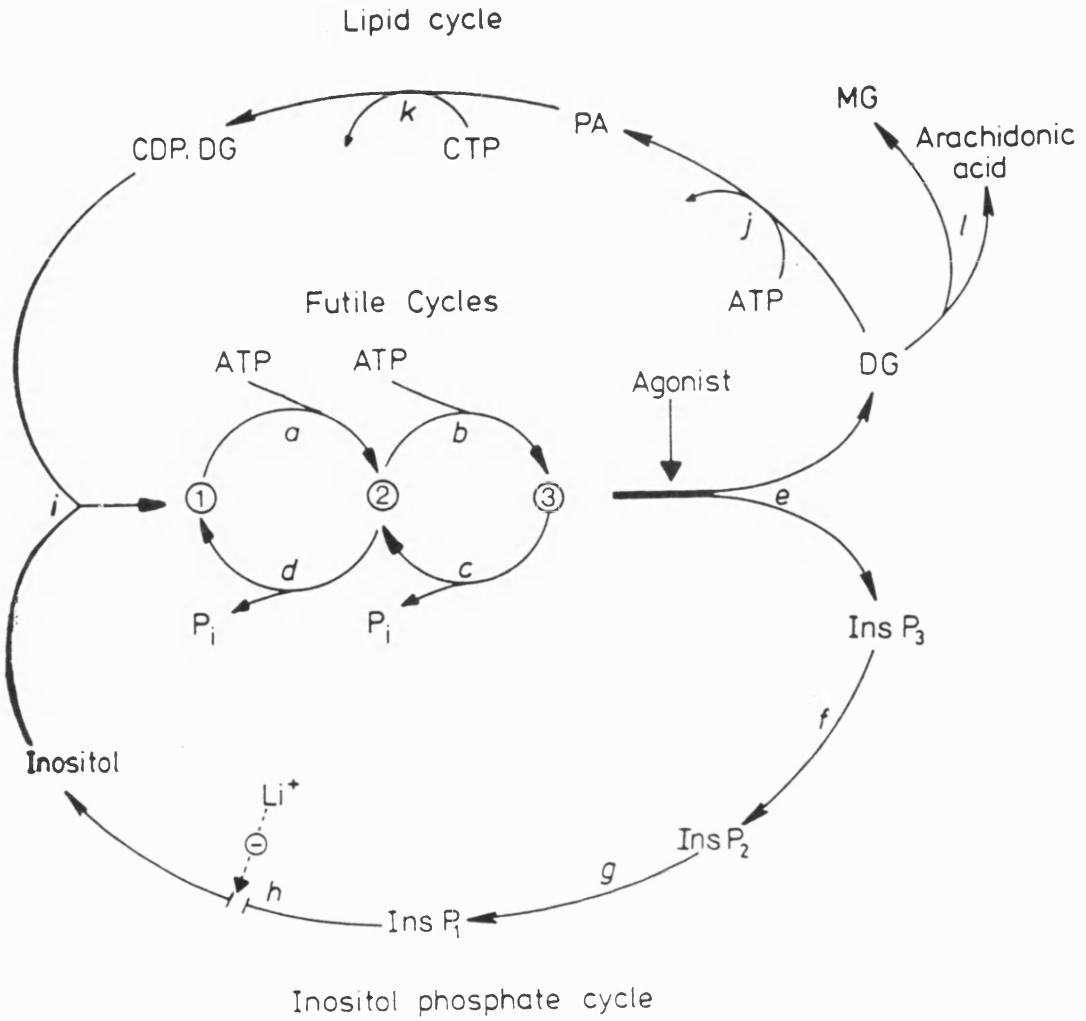


Fig. 1.3. Inositol Lipid Metabolism and Signal Transduction

An inositol phosphate cycle converts IP_3 to free inositol which interacts with CDP-DG to reform PI. The CDP-DG is the product of a lipid cycle which channels DAG back to PI. *a*, PI kinase; *b*, PIP kinase; *c*, PIP_2 phosphomonoesterase; *d*, PIP phosphomonoesterase; *e*, PIP_2 phosphodiesterase; *f*, IP_3 phosphatase; *g*, IP_2 phosphatase; *h*, IP_1 phosphatase; *i*, CDP-DAG inositol phosphatidate transferase; *j*, DAG kinase; *k*, CTP phosphatidate cytidyl transferase; *l*, DAG lipase. PA, phosphatidic acid. 1, PI; 2, PIP; 3, PIP_2.

phosphatase effectively terminates Ca^{2+}-mobilization by Ins(1,4,5)P_3. Receptor mediated increases in IP_3 are often transient despite sustained increases in total inositol phosphate production; so, following cell stimulation, IP_3 metabolism may be accelerated by regulation of the phosphatase (or kinase). PKC has been implicated in this process (Connolly *et al.*, 1987) as phorbol esters activated 5-phosphatase activity in pretreated platelets, as has a receptor-mediated increase in [Ca^{2+}] (Kukita *et al.*, 1986). Neither theory has been universally accepted (Shears, 1989). The Ins(1,4,5)P_3 derived Ins(1,4)P_2 is further dephosphorylated by two independent routes to form Ins(1)P_1 and Ins(4)P_1 (Ackermann *et al.*, 1987; Bansal *et al.*, 1990). The two monophosphates are then hydrolysed to form free inositol. Evidence suggests that the same enzyme is responsible for the breakdown of all the inositol monophosphate isomers (Ackermann *et al.*, 1987; Shears, 1989) for which it has similar affinities.

Ins(1,4,5)P_3 may alternatively be phosphorylated in the 3-position by an ATP-requiring kinase that is highly specific (Connolly *et al.*, 1987) and soluble in most tissues examined (Irvine *et al.*, 1986). The enhancement of Ins(1,4,5)P_3 metabolism following receptor activation is probably largely due to receptor-mediated activation of the kinase. This may sometimes be affected by PKC, and more frequently by increases in Ca^{2+} that act in a calmodulin dependent manner. Ins(1,3,4,5)P_4 is hydrolysed by a specific 5-phosphatase reaction to form Ins(1,3,4)P_3 (Batty *et al.*, 1985; Downes *et*

al., 1986). It is possible that the same enzyme may be responsible for the dephosphorylation of both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, but if both molecules have second messenger characteristics then their concentrations and subcellular distributions are most likely to be controlled by a number of different isoenzymes with varying substrate specificities, kinetic parameters and subcellular locations (Hansen *et al.*, 1987). Ins(1,3,4)P_3 is dephosphorylated to Ins(1,3)P_2 and Ins(3,4)P_2 (Inhorn *et al.*, 1987), the latter being the major product (Hansen *et al.*, 1986). The degradative pathways of the IP_2 isomers have not been fully elucidated. The two possible IP_1 products of Ins(1,3)P_2 metabolism are stereoisomers unresolved by non-chiral HPLC techniques but the 3-phosphatase attack on Ins(1,3)P_2 has been characterised (Bansal *et al.*, 1987).

1.4.1.1. Other Routes of Inositol Phosphate Metabolism

Balla *et al.* (1987) reported that three inositol tetraphosphates increased in angiotensin-stimulated adrenal glomerulosa cells. One of these was identified as Ins(1,3,4,5)P_4, while another appeared to be the product of a kinase enzyme that acts on Ins(1,3,4)P_3 to form Ins(1,3,4,6)P_4. The formation and subsequent metabolism of this compound is currently unknown. The third isomer detected in adrenal cells is of unknown structure. Harden *et al.* (1987) labelled turkey erythrocytes by incubation with $^{32}PO_4$. They detected Ins(1,3,4,5)P_4 and Ins(1,4,5,6)P_4, and kinetic data inferred that although Ins(1,4,5,6)P_4 was

present in higher concentrations, it was metabolically inert.

IP_5 and IP_6 were among the first metabolites to be discovered and yet very little is known about their metabolism in animal cells, which occurs through routes that are largely separate from the agonist-sensitive pathways. In the case of IP_5, a pathway exists from agonist-sensitive inositol phosphate through the phosphorylation of Ins(1,3,4)P_3 to Ins(1,3,4,6)P_4 and then to Ins(1,3,4,5,6)P_5 (Stephens *et al.*, 1988). The main route of synthesis however is probably through Ins(3,4,5,6)P_4 which has long been known to be a constituent of avian erythrocytes (Johnson & Tate, 1969) where it is thought to control the oxygen affinity of haemoglobin. The origin of Ins(3,4,5,6)P_4 is as yet unknown - it may be formed by phosphorylation of inositol or indirectly from Ins(1,4,5)P_3, and it is not known if other IP_5 isomers exist in different cells. Synthesis of IP_6 (phytic acid) is thought to be through the phosphorylation of inositol, but the full pathway is not known. The levels of IP_5 and IP_6 do not alter greatly after agonist stimulation and they are thought to have housekeeping functions rather than operate as second messengers. IP_6 may have a selective cytotoxin role in plants (Mayr, 1989) and it has been suggested that IP_5 and IP_6 have extracellular functions, but their intracellular properties have not been clarified.

1.4.1.2. Cyclic Inositol Phosphates

The family of Ins 1:2-cyclic phosphates may be normal constituents of the cell, though their role during acute cellular stimulation is still unclear. The proportion of cyclic inositol phosphates produced in various cells is unknown mainly because of technical difficulties in measuring these acid-labile substances. Ins(1:2 cyc, 4,5)P_3 is produced following the receptor mediated hydrolysis of PIP_2 but is thought to comprise less than 5% of the total trisphosphate released (Berridge, 1989). It is dephosphorylated to Ins(1:2 cyc, 4)P_2, and thus to Ins(1:2 cyc)P without cleavage of the 1:2 cyclic phosphate bond. Ins(1:2 cyc)P is cleaved to form Ins(1)P_1 by Ins(1:2 cyc)P 2-phosphohydrolase (Majerus *et al.*, 1986; Majerus, 1992) which is finally converted back to free inositol. The hydrolase does not affect cyclic inositol polyphosphates and appears to be uninhibited by lithium. The degradation of cyclic IP_3 is much slower than that of non-cyclic IP_3, and it is not a substrate for IP_3 kinase (Downes *et al.*, 1989). Thus far, there is no evidence for enzymatic interconversion of the cyclic and non-cyclic inositol polyphosphates in cell extracts.

1.4.2 1, 2-*sn* Diacylglycerol

DAG evolved during PLC-mediated breakdown of inositol phospholipids appears to have at least 2 metabolic fates, following the action of a kinase or a lipase. In the former

case DAG is phosphorylated to phosphatidic acid by 1,2-DAG kinase. This enzyme appears to be present mainly in the plasma membrane but can also occur in a soluble form (Lapetina & Hawthorne, 1971). Phosphatidic acid (PA) then combines with cytosine triphosphate (CTP) to form the high energy intermediate CMP-phosphatidate which accepts a free inositol molecule to reform PI. The fatty acid part of the molecule is thus conserved and the PI can be reused for signalling. This reaction occurs in the endoplasmic reticulum (ER), but does not readily account for the total reconversion of phosphatidic acid to PI. PA is the precursor for glycerolipids and the recycling process relies on the occurrence of pools of metabolically segregated inositol phospholipids (Downes *et al.*, 1989).

Alternatively, DAG is hydrolysed by a monoacylglycerol lipase to release arachidonic acid for the formation of prostaglandins in mammalian cells. Most of the arachidonic acid in cells is esterified to glycerophospholipids from which it is released by various phospholipases. The release of arachidonic acid by phospholipase A_2 may be regulated by both limbs of the PIP_2 turnover pathway - IP_3 may cause the release of calcium to activate the lipase, whilst the DAG/PKC limb may phosphorylate and hence inactivate a protein termed lipocortin, which inhibits phospholipase A_2 (Berridge, 1987b).

1.5 $Ins(1,4,5)P_3$ Induced Calcium Mobilization

When cells respond to calcium-mobilizing agonists that act through the hydrolysis of inositol phospholipids, they draw upon both intracellular and extracellular sources of calcium. The latter remains relatively constant but the distribution and size of the internal pools varies greatly (Berridge and Irvine, 1989; Berridge, 1991). Of all the inositol phosphates that have been identified in cells, $Ins(1,4,5)P_3$ is the only one so far for which a clear second messenger role has been defined. The first direct evidence to show intracellular calcium release from a non-mitochondrial pool in response to $Ins(1,4,5)P_3$ was obtained using permeabilized pancreatic cells (Streb *et al.*, 1983). Using a calcium electrode it was possible to monitor the uptake of calcium into the ER and its subsequent release following addition of the trisphosphate. To release calcium, $Ins(1,4,5)P_3$ must bind to receptors that are somehow linked to calcium channels connected with the $Ins(1,4,5)P_3$-sensitive calcium pool. Only part of the pool is $Ins(1,4,5)P_3$-sensitive; on average 30-50% of the calcium taken up by the non-mitochondrial pool is released, the remainder can be mobilized by ionophores. The insensitive pool may be a separate membrane compartment possessing distinct calcium-pumping properties. Once bound, the channel opens and calcium leaks into the cytosol by passive efflux from the specific membrane pool. Quantal flux of Ca^{2+} elicited by IP_3 is however a fundamental property of its receptor (Ferris *et al.*, 1992) and IP_3 induced calcium

release has been shown to be a steady-state response controlled by luminal Ca^{2+} (Missiaen *et al.*, 1992).

The cellular location and identity of the sensitive and insensitive pools are uncertain but it is thought that the ER immediately adjacent to the plasma membrane is sensitive to $Ins(1,4,5)P_3$, whereas that further within the cell is insensitive (Berridge, 1987a). Volpe *et al.* (1988) also suggested the calciosome as a sensitive pool in peripheral tissues, a small membrane vesicle that has properties characteristic of muscle sarcoplasmic reticulum (SR). Muscle SR has IP_3-gated Ca^{2+} channels which differ in conductance and pharmacology from those found in aortic SR for example (Erhlich and Watras, 1988). Calciosomes contain the calcium binding protein sequestrin and have muscle-like calcium pumps, but their relationship to the trisphosphate sensitive or insensitive pools is unclear.

Of the inositol phosphates tested, only those having phosphates on the 4- and 5- positions are capable of stimulating release of calcium (Irvine *et al.*, 1984b) whereas the phosphate on the opposite side of the molecule (1- position) functions to enhance the affinity of the molecule for its receptor.

Although there is quite convincing evidence for the mobilization of internal Ca^{2+}, the second phase of the Ca^{2+} response, Ca^{2+} entry is not well understood. Evidence has been collected to suggest that inositol lipid metabolism is

important for calcium entry. Microinjection of inositol phosphates including IP_3 into sea urchin eggs or lacrimal cells resulted in cell responses requiring both Ca^{2+} release and Ca^{2+} entry from the extracellular space (Irvine and Moor, 1986; Morris *et al.*, 1987). Direct application of Ins(1,4,5)P_3 to plasma membranes does not however increase their permeability to calcium. These observations suggest that Ins(1,4,5)P_3 may activate calcium influx into cells, but not by a direct action at the plasma membrane. Putney Jr. (1986) suggested a capacitance mechanism in which Ins(1,4,5)P_3 secondarily promotes Ca^{2+} entry by emptying intracellular Ca^{2+} stores. Under resting conditions the IP_3-sensitive intracellular Ca^{2+} store is resistant to depletion by extracellular chelating agents, but once depleted, refilling only occurs in the presence of extracellular Ca^{2+} and after the removal of the first stimulus (Hughes and Putney, 1989). Emptying of the intracellular Ca^{2+} pool by IP_3 allows direct communication of this pool with the plasma membrane. In the presence of extracellular Ca^{2+}, calcium enters the cell through this interface and then passes into the cytosol. When IP_3 is degraded, extracellular Ca^{2+} continues to enter the cell *via* this interface until the intracellular Ca^{2+} pool is restored.

Another hypothesis for linking inositol lipids to calcium entry across the plasma membrane also involves inositol polyphosphates. Preliminary evidence suggests that Ins(1,3,4,5)P_4 may function to control calcium entry (Morris *et al.*, 1987; Houslay, 1987; Neher, 1992). Following calcium

release by Ins(1,4,5)P_3, the trisphosphate is converted to Ins(1,3,4,5)P_4 which would function to control subsequent calcium entry from extracellular stores. Since the conversion of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 is enhanced by calcium, stimulation of influx by this IP_4 would be an example of calcium-induced calcium entry. Evidence for this theory was provided when the injection of Ins(1,4,5)P_3 into sea urchin eggs resulted in cell activation which was believed to require both Ca^{2+} release and entry (Irvine and Moor, 1986).

It was demonstrated that the injection of Ins(2,4,5)P_3, which releases intracellular Ca^{2+} but is not phosphorylated to IP_4, resulted in the activation of many fewer cells. Microinjection of Ins(1,3,4,5)P_4 alone failed to elicit a response but injection together with Ins(2,4,5)P_3 produced a full response. The activation of Ca^{2+} entry was thought to require phosphorylation of IP_3 to IP_4 and emptying of the intracellular Ca^{2+} pool. Alternatively, the DAG formed by the hydrolysis of PIP_2 is phosphorylated to PA which may then act as a calcium ionophore before it is resynthesized to PI.

The phosphoinositide signalling system is not uniformly distributed throughout the cell (Cheek, 1989). Nicotine acts by depolarizing the membrane, which then leads to the opening of voltage-operated calcium channels and the influx of external calcium. Initially the calcium is present as a halo adjacent to the membrane but it later develops into a

more widespread response. Conversely, muscarinic stimulation results in a more localised response, possibly as a result of the Ins(1,4,5)P_3-sensitive store being concentrated in one particular area (Berridge, 1989). In the former example, the mechanism of the wave-like spread of calcium is still unclear, but it is thought not to be by diffusion alone. Berridge (1989) and Berridge and Irvine (1989) have suggested that Ins(1,4,5)P_3-insensitive calcium stores are responsible for triggering the wave, which then propagates by a process of calcium-induced calcium release coupled with diffusion. Ins(1,4,5)P_3-released calcium from sensitive pools diffuses towards insensitive pools and triggers further calcium release. This diffuses to another insensitive pool and the reaction continues. The precise form and dynamics of calcium waves will depend on how the stores are organised, and in some systems can spread from cell to cell.

The intracellular level of calcium often oscillates, especially when the phosphoinositide pathway is being stimulated (Berridge, 1990, 1992). These oscillations can take many forms but are usually expressed as transient calcium 'spikes' of increased concentration separated by variable intervals where the level remains at or close to resting levels. The basic feature of these oscillations is that they are triggered by the periodic release of stored calcium and not voltage-operated channels in the plasma membrane. Whereas the form and frequency of the oscillations vary from cell to cell, they are very consistent for

individual cells responding to a specific agonist (Prentki *et al.*, 1988). IP_3 is almost universally implicated as a key element in the oscillatory mechanism - IP_3 fluctuations may set up negative feedback interactions acting on PLC, or calcium may act positively to create periodic surges of IP_3 with the concomitant release of calcium amplified to produce a full spike. For the Ins(1,4,5)P_3-sensitive pools to refill, Ins(1,4,5)P_3 and calcium must be removed. A model to explain how a steady level of Ins(1,4,5)P_3 can set off calcium oscillations is based on the existence of separate calcium pools (Berridge, 1989; Wakui *et al.*, 1992). Calcium released by the Ins(1,4,5)P_3-sensitive pool functions to prime and then to trigger calcium release from the Ins(1,4,5)P_3-insensitive pool by a process of calcium-induced calcium release - the same model proposed for calcium waves.

The distribution of calcium signals in space and time seem to be reflections of a common mechanism (Berridge, 1988, 1989; Berridge and Irvine, 1989). A unified hypothesis suggests that each transient during a calcium oscillation can be spatially organised, as it initiates at a specific point and spreads in a wave-like manner through the cell. The agonist-stimulated release of Ins(1,4,5)P_3 activates the process of calcium-induced calcium release that is responsible for the spatially organised calcium transient. Although the significance of the different components may vary from cell to cell, both oscillations and signal

propagation may share a common mechanism (Berridge, 1992; Dupont *et al.*, 1991).

1.6 Yeast as an Investigative Model

If the phosphatidylinositol signal transduction system is fundamental to mammalian cells and has ancient origins, then it may exist and operate in simple eukaryotes although the regulatory roles and functions may be different. Yeast, particularly strains of *Saccharomyces cerevisiae* were selected as a representative organism for the investigation as they provide convenient models for most of the activities of cells of higher forms of life (Campbell and Duffus, 1988). Yeast is also safe to handle and can be grown easily. The project has, as its prime focus, the biochemistry of the signalling pathway, but any biochemical work could be readily complemented with a vast array of genetic experiments. Many of the genetic techniques were developed specifically for yeast, and some procedures which are not possible in higher cells, can be readily performed in this organism. Considering experimental factors, metabolism, genetic and developmental responses can be elicited under well defined conditions, facilitating reproducibility and reliability. Should the need arise, yeasts can be grown as haploid or diploid cells, or indeed as higher ploidies if required.

1.7 Evidence for the Transduction System in Yeast

Primary evidence for the existence of a phosphoinositide second messenger system in yeast includes the detection and isolation of the membrane lipids PI, PIP and PIP_2 (Steiner and Lester, 1972a; Lester and Steiner, 1968; Prottey *et al.*, 1970). Subsequently, Wheeler *et al.* (1972) demonstrated enzymes capable of catalyzing the synthesis of these compounds including a kinase responsible for the incorporation of exogenous [Γ^{32}P] ATP into PIP_2 located in the membrane. Belunis *et al.* (1988) purified PI kinase from yeast cell membranes and showed an activity which was associated with a 35,000 Da membrane-associated protein. The product of the reaction catalysed by the purified enzyme was shown to be phosphatidylinositol-4-phosphate (PI(4)P). It was originally thought that the phosphorylation of PI only involved the production of PI(4)P and PI(4,5)P_2 but Auger *et al.* (1989a) demonstrated that yeast cell membranes also contain phosphatidylinositol-3-phosphate (PI(3)P), and identified a specific membrane-associated enzyme which catalysed the synthesis of PI(3)P from PI. Similar activities had only recently been discovered in mammalian cells (Kaplan *et al.*, 1987; Whitman *et al.*, 1988) and led to the discovery of a new PIP_2 isomer, PI(3,4)P_2 (Auger *et al.*, 1989a) and the novel polyphosphoinositide, phosphatidylinositol trisphosphate (PIP_3), (Traynor-Kaplan *et al.*, 1988; Auger *et al.*, 1989b and Stephens *et al.*, 1991). In contrast to mammalian cells, the experiments of Auger *et al.* (1989a) suggested that the steady state level

of PI(3)P *in vivo* made up 50% of the total PIP in the membranes of yeast cells. This implied that PI kinase activity in yeast must be heterogeneous and consists of both PI(3)P and PI(4)P kinases. It also suggested that the enzymes purified thus far could just be some of several enzymes capable of catalysing PI conversion. The potential role of PI(3)P as an effector in signal transduction was reviewed by Downes and Carter (1991) and recent developments in polyphosphoinositide investigation were reviewed in Carpenter and Cantley (1990), Downes and Macphee (1990) and Hawkins *et al.* (1992a).

PI kinase activity is reputedly sensitive to growth phase and cAMP concentrations within the cell (Holland *et al.*, 1988; Kato *et al.*, 1989). The conclusions reached by these groups of researchers are however in disagreement. Holland *et al.* (1988) demonstrated that PI kinase activity increased 2-2.5 fold as cells entered the stationary phase, and this correlated with a large drop in cAMP levels. Using mutant yeast strains, Kato *et al.* (1989) showed the opposite result. Extracts prepared from strains containing *ras1*, *ras2* or *cyr1* mutations (known to affect cAMP-dependent phosphorylation) showed PI kinase and PIP kinase activities to be 30-60% lower than wild type extracts assayed under the same conditions. Kato *et al.* (1989) concluded that phosphoinositide kinase activity is elevated under conditions which favour cAMP-dependent protein phosphorylation and Holland *et al.* (1988) reached the opposite conclusion. This may have been due to the use of

different strains, growth and assay conditions but may also reflect the potentially heterogeneous population of kinase enzymes in yeast. It is possible that the separate groups were looking at entirely different enzymes, and data on the regulation of specific individual PI kinases are not yet available.

Other relevant metabolites that have been detected in yeast include the calcium-binding protein calmodulin (Davis and Thorner, 1986; Davis *et al.*, 1986). This implies a calcium signalling pathway is present. Cloning and characterization of genes for two calcium-binding proteins, calmodulin and the *CDC31* gene product have demonstrated that both of these proteins perform vital, but different functions in yeast. Kaibuchi *et al.* (1986) stated that when yeast cells were grown in media containing 2% glucose and then starved in a buffer containing 0.02% glucose they arrested at the G_0/G_1 phase of the cell cycle; but also showed that the addition of glucose relieved the cell-cycle arrest and instigated the rapid incorporation of $^{32}P_i$ into PI, PIP and PIP_2 in a time-dependent manner. This was reputedly accompanied by the production of radioactive $Ins(4)P_1$ and the second messenger $Ins(1,4,5)P_3$, presumably *via* the action of phospholipase enzymes. Evidence was however provided for the presence of inositol phosphates in yeast. The existence of a protein kinase C (PKC) in yeast has been suggested recently and was initiated by detection of a protein kinase dependent on Ca^{2+} and calmodulin in *Sacch. cerevisiae* (Miyakawa *et al.*, 1989) and the cloning of a gene (Levin *et al.*, 1990) with some

homology to mammalian PKC (*PKC1*). Ogita *et al.* (1990) then purified an activity which was related to the mammalian enzyme but was neither activated by the phorbol ester TPA, nor efficiently phosphorylated lysine-rich histones (H1). Simon *et al.* (1991) isolated a different PKC-like enzymatic activity from *Sacch. cerevisiae* using a different purification scheme. This enzyme was purified using chromatography on DEAE-cellulose followed by hydroxylapetite which separated the activity into three distinguishable sub-species, analogous to the mammalian PKC isoenzymes. The fractions enriched in PKC activity contained proteins that specifically bound TPA, were specifically phosphorylated in the presence of DAG and recognised by anti-mammalian PKC antibodies. More recently, the same group has isolated three different mammalian-like PKC clones through the screening of yeast expression libraries (Simon *et al.*, 1992) and the predicted amino acid sequence for one of the clones, yPKCII is highly homologous to the C1 region (the DAG binding domain) of the mammalian α, β and Γ PKC isoenzymes. These results suggest the existence of a family of related mammalian-like PKC genes in *Sacch. cerevisiae.*

Of a more circumstantial nature, when inositol-requiring mutants of *Sacch. cerevisiae* are deprived of inositol under conditions that are otherwise growth supporting, rapid biochemical changes occur leading to inositol-less death (Henry, 1982). In contrast, mutants auxotrophic for many other compounds including amino acids or choline, stop dividing and lose viability relatively slowly when starved

for their requirement (Henry *et al.*, 1977). This implies that inositol plays a vital role in maintaining the cell viability and since inositol-requiring mutants rapidly cease production of PI under conditions of inositol deprivation, whilst macromolecular synthesis continues unimpeded (Henry *et al.*, 1977), that role may be associated with the membrane phosphoinositides. Although this has been known for several decades, the mechanism of inositol-less death is not understood (White *et al.*, 1991) but it may occur because the cessation of synthesis of essential components of the cell wall and plasma membrane is not coupled with an orderly cessation of overall cellular metabolism. In normal growth conditions the proportion of PI in yeast plasma membranes remains particularly stable, being the least alterable of the lipid constituents and retaining a high degree of saturation (Cartwright *et al.*, 1987). This also suggests an important role for inositol containing phospholipids as does the fact that PI is the only membrane component whose level falls dramatically after the arrest at 'START', the major regulatory point for proliferation in yeast cells (Wheals, 1987), *via* either mutation or G1 inhibition (Dudani *et al.*, 1983; Dudani and Prasad, 1984). In addition, PI undergoes rapid turnover (Hokin and Hokin, 1964) indicating a high level of metabolic activity.

Direct evidence for a yeast phosphoinositide system includes the results of Kaibuchi *et al.* (1986) who showed that the addition of glucose to resting cells stimulated PIP_2 turnover. Also, Uno *et al.* (1988) reported that when a

monoclonal antibody specific for PIP_2 was introduced into yeast cells by electroporation it caused arrest of cells at the G_0/G_1 phase of the cell cycle. They concluded that PIP_2 plays a role in the transition through the cell cycle, probably mediated through a response to glucose. During this work five genes were detected (*PIM1-5*), mutations in which cause defects in PI metabolism and cell cycle arrest. Two of the mutants produced were defective in PI kinase (*pim2*) and PIP kinase (*pim1*) activity, but cell cycle arrest in these cells could be relieved by the introduction of PIP_2 by electroporation or the combination of IP_3 and a synthetic form of DAG. Recently it was found that *PIM3*, *PIM4* and *PIM5* were allelic to *RAS2*, *CYR1* and *BCY1* (Ishikawa, T., 1990; pers. comm.), the mammalian genes involved in the PI transduction system. Londesborough and Nuutinen (1987) demonstrated the presence of a protein kinase with calcium ion/calmodulin activation dependency.

The natural yeast sterol, ergosterol stimulates phosphoinositide turnover and cell proliferation under defined conditions in an ergosterol-requiring strain (Dahl and Dahl, 1985). In "shift-up" experiments using an inositol-requiring strain, an initial increase of ^{32}P-labelling of PI, PIP and PIP_2 was recorded when cells grown in cholesterol were shifted to ergosterol-containing media. The most significant increase was in PIP_2, and most rapid turnover was also noted for this phospholipid. Dahl *et al.* (1987) showed that ergosterol can also stimulate PI kinase activity and suggested that it may be a regulator of enzyme

activity in addition to its structural role in membranes as a bulk lipid.

Further support that the PI transduction system may have an ancient origin and therefore be present in simple eukaryotes derives from a PI system described in *Neurospora crassa* (Hanson, 1991) and an inositol lipid signalling pathway in the yeast-mycelium transition of the dutch elm disease fungus, *Ophiostoma ulmi* (Brunton and Gadd, 1991). Structural and functional homologues to human *ras* genes have been discovered in yeast (Defeo-Jones *et al.*, 1983; Powers *et al.*, 1984) and encode proteins involved in the stimulation of adenylate cyclase (Tamanoi, 1988; Broach, 1991; Wheals, 1985). Kaibuchi *et al.* (1986) suggested that PI turnover is affected by *RAS* genes. Higher rates of PI turnover were noted in *ras1* and *ras2* mutants suggesting a regulatory role for RAS1 and RAS21 proteins.

Based on what would appear to be a quite substantial collection of evidence, it seems reasonable to propose that a phosphatidylinositol signal transduction system, comparable to that in mammalian cells, exists and operates in *Saccharomyces cerevisiae.*

1.8 Signal Transduction in *Saccharomyces cerevisiae*

Response to pheromones during yeast mating provided an opportunity to study signal transduction in a unicellular eukaryote - extracellular peptide mating pheromones activate

a pathway through membrane protein receptors that leads to cellular differentiation in preparation for mating, which is manifested by transcriptional induction of numerous genes, by morphological changes and cell division cycle arrest at G1 (Marsh *et al.*, 1991). Haploid yeast cells of either mating phenotype, **a** and *α* can proliferate by mitotic cell division but also have the potential to form an **a**/*α* diploid. Mating is the culmination of a 2-way signalling process between **a** and *α* cells. Each haploid cell type secretes a unique signalling molecule, **a**-factor or *α*-factor, and displays at its surface a receptor that can bind the pheromone secreted by the opposite mating type. Binding activates an intracellular signal transduction pathway that is common to **a** and *α* cells (Sprague, Jr., 1991). As a result of the cellular differentiation, cell and nuclear fusion can proceed to produce the **a**/*α* diploid.

The intracellular response pathway is identical in both cell types, all mutations that affect the response of **a**-cells to *α*-factor also influence the response of *α*-cells to **a**-factor except mutations in the receptor structural genes. Also, an **a**-cell engineered to express the *α*-factor receptor will respond to **a**-factor, not *α*-factor. The reverse of this can be shown in *α*-cells (Bender and Sprague, Jr., 1986). Both receptors are coupled to the same heterotrimeric G protein ($G_{\alpha\beta\Gamma}$), the α subunit of which is possibly encoded by GPA1 (Miyajima *et al.*, 1987). When pheromone binds, the receptor undergoes a conformational change that activates the G-protein and releases the βΓ subunit (potentially encoded by

STE4 and *STE18*, Whiteway *et al.*, 1989). This activates downstream components of the pathway. The immediate target of the G protein is not known but four serine/threonine protein kinases that are required for signal transmission have been identified (the products of the STE11, STE7, FUS3 and KSS1 genes) and recent evidence indicates that the activity of one or more of these kinases is increased when the pathway is stimulated (Sprague, Jr., 1991; Marsh *et al.*, 1991). Thus, a phosphorylation cascade appears to be an essential feature of the pathway. Ultimately a transcriptional activator, STE12 gene product, is activated which leads to differentiation - increased transcription of several genes including those encoding cell-surface proteins involved in cell-cell interactions and fusion. Because the production of these proteins is part of the differentiation that accompanies pheromone stimulation, the STE12 gene product is thought to carry out the terminal step of the pathway. A second target for kinase activity may be the G1 cyclins, proteins required for the activity of the Cdc28 protein kinase (required for progression from G1 to S phase). Upon pheromone stimulation, the cyclins are inactivated and hence the cells arrest in G1.

The existence of a signal transduction system in yeast has been well established, and information has been gathered relevant to the initial receptor-ligand interaction through to the final differentiation and cell cycle arrest. There are still significant gaps in the understanding of the system but it was found that many of the intermediates of

the pathway, in addition to the receptors and the G-proteins, had mammalian homologues. For example *FUS3* and *KSS1* gene products are homologous to *ERK1* kinase which is thought to be involved in insulin stimulated signal transduction (Boulton *et al.*, 1990; Pawson, 1991). If information gained from the investigation of this pathway is applicable to higher eukaryote physiology, it is possible that the results gained from a study of the putative PI transduction system would also be relevant to the established mammalian system. Recent reviews of the pheromone signalling pathway and its mechanism include those by Herskowitz (1989), Kurjan (1990) and Sprague, Jr. (1990).

1.9 Project Aims

The principal aim of the project was to establish whether a PI signal transduction system existed and operated within *Saccharomyces cerevisiae*, and to determine the role of this system in controlling cellular responses to environmental and developmental influences; such as changes in cell proliferation rate, initiation of sporulation, alteration of the size control and mating.

The objectives to achieve this aim were concerned with the important metabolite inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$). The ability to demonstrate the presence of $Ins(1,4,5)P_3$ would provide substantial evidence for the existence of the secondary messenger pathway. Hence, the investigation was directed at $Ins(1,4,5)P_3$ in 3 ways.

Firstly, the detection and isolation of Ins(1,4,5)P_3 from a yeast cell extract, which would involve the development of a sensitive and reliable assay. Secondly, the production of IP_3 from exogenously added PIP_2, by incubating the precursor with whole or broken yeast cells and a stimulant. This would demonstrate the activity of phospholipase C and support further the existence of a transduction system in yeast. Thirdly, the breakdown of Ins(1,4,5)P_3 using cellular material would demonstrate degradative enzymes that comprise the pathway that recycles the inositol phosphates back to free inositol.

Such demonstrations would show that not only is Ins(1,4,5)P_3 present in yeast, it is produced *via* the receptor-mediated hydrolysis of PIP_2 and is then deactivated in the cytosol, and recycled back to inositol. This would corroborate the system existed and operated in yeast.

The project is concerned with the biochemical aspects of the pathway and although a large number of intermediates are involved in the PI system, assays were to be developed primarily for the water-soluble inositol phosphates, such as IP_3 and IP_4, which would act as key markers for investigating the system. Having devised an assay, analysis of the inositol phosphates was to be performed by High Performance Liquid Chromatography (HPLC), which can also give information relating to other phosphoinositides (Berridge, 1983; Irvine *et al.*, 1982). The development of an

inositol phosphate assay was hampered by a number of factors - neither inositol nor its phosphates absorb in the ultra-violet or visible spectra, so detection by these methods was impossible. It was however possible to use radiolabelled tracers and monitor turnover by liquid scintillation spectrometry.

The assays were used to measure the concentration or turnover of the relevant metabolites. The changes in these parameters could then be measured during developmental modifications induced by environmental conditions. These may include altering the carbon or nitrogen source, or altering growth rate during the initiation of sporulation, during mating pheromone cell proliferation arrest and after a temperature shift with temperature-sensitive mutants affected in any of these processes.

1.10 Proposed Methodology

The original intention was to grow yeast cells in the presence of radioactive tracer inositol and a stimulating factor - to initiate the putatively present transduction system. After incubation, the cells were to be harvested and water soluble metabolites extracted for analysis. A wild type strain (A364A) was selected as the starter organism and grown in a complex medium with tritiated inositol. Following a 24 h incubation the yeasts were to be harvested in such a way that the required compounds would be readily available for separation and identification. The growth and extraction

methodologies were altered many times to improve the recovery of radioactivity *per se*, and radioactive inositol phosphates. Details are included in the text. The separation and identification of inositol phosphates was performed by anion exchange column chromatography.

1.11 Ion Exchange Chromatography

The principal feature of ion exchange chromatography (IEC) is the attraction between oppositely charged particles. Biological materials, such as amino acids and proteins have ionisable groups, and possess a net positive or negative charge which can be used for separating mixtures of such compounds (Wilson and Goulding, 1986).

Ion exchange was performed in columns packed with an insoluble solid material that carried exchangeable cations or anions. Anion exchangers, as used in this project have positively charged groups which attract negatively charged molecules. The exchangeable ions or 'counter ions' are replaced by sample ions that have the same charge; neutral species and cations do not interact, and pass through the column. The required ions, once stuck to the column, can be removed or 'eluted' by introducing another ion of higher affinity for the exchange site. In general, the lower the selectivity of the ion, the more readily it exchanges for another ion of like charge (Bio-Rad, Strong Anion Exchange Resin Instruction Manual).

The first anion exchanger used was Dowex resin (Bio-Rad, Herts., UK), a strongly basic anion exchanger, capable of exchanging anions of basic, acidic and neutral salts. The AG1-X8 resin used is loaded with quaternary ammonium functional groups attached to a styrene divinylbenzene copolymer lattice with cross-linkage of 8%.

Once the elution system was checked with Dowex resin chromatography, the separation of phosphates could be upgraded to HPLC. The same principles were involved, but the column support matrix (stationary phase) consisted of a more finely divided particulate material. This was physically much stronger and could therefore withstand higher pressures allowing much better separations to be achieved in a shorter time.

2.0 Materials and Methods

2.1 Strains Used

A364A

Genotype MATa *ade1 ade2 ura1 his7 lys2 tyr1 gal1.*
This is a well characterised yeast strain with known markers that was used extensively in cell cycle work by Hartwell (1967, 1976), Hartwell *et al.* (1970, 1973), Culotti and Hartwell (1967) and Bucking-Throm *et al.* (1973).

SB4

Genotype MATa *ade2 ura3 can1 met13 leu1-12 ino1 ino4.*
This is an inositol requiring mutant, obtained from J.R.Dickinson (U.W.C.C.) intended for use in uptake-of-radioactivity experiments. Banerjee (1984) describes SB4 as non-auxotrophic for inositol but experiments with inositol +/- media and drop out plates confirmed all markers shown above, including temperature sensitivity for *pfk* at 36 ^{o}C on complex media.

MC3

Genotype MATa *ino1-13 ino2-8 gal2 can1.*
This is an inositol requiring mutant obtained from the Yeast Genetic Stock Centre, University of California, Berkeley.

Strains were maintained on YEPD slopes, YEPE and YEPG plates at 4 ^{o}C and under nitrogen at -20 ^{o}C.

2.2 Media Composition

YEPD (Yeast Extract, Peptone, D-glucose)

Bacto-Yeast Extract (MC1) LabM, 1 % (w/v).

Bacto Peptone (MC9, Mycological) LabM, 2 % (w/v).

D-glucose, BDH, Poole, Dorset, UK, 2 % (w/v).

Difco Agar, 2 % (w/v, added for solid media) .

YEPE (Yeast Extract, Peptone, Ethanol)

Difco Yeast Extract, 1 % (w/v).

Difco Peptone, 2 % (w/v).

Ethanol (100 %), filter sterilised, 1 % (v/v).

Difco Agar, 2 % (w/v, added for solid media).

YEPG (Yeast Extract, Peptone, Glycerol)

Difco Yeast Extract, 1 % (w/v).

Difco Peptone, 2 % (w/v).

Glycerol, 3 % (v/v).

Difco Agar, 2 % (w/v, added for solid media).

Adenine (0.1 g l^{-1}) and uracil (0.1 g l^{-1}) could also be added to the above media to supplement adenine and uracil requiring mutants.

Minimal Medium (Glucose Minimal)

Bacto Yeast Nitrogen Base Without Amino Acids, 0.67 % (w/v).

D-glucose, 4 % (w/v).

Adenine 20 mg l^{-1}, uracil 20 mg l^{-1}.

Amino acids:- tyrosine 30 mg l^{-1}, histidine 20 mg l^{-1},

lysine 30 mg l^{-1}, tryptophan 20 mg l^{-1} and threonine 200 mg l^{-1}.

Difco Agar, 2 % (w/v, added for solid media).

Defined Medium

Difco Vitamin Free Yeast Base, 0.67 % (w/v).

D-glucose, 2 % (w/v).

Vitamins:- biotin 20 µg l^{-1}, calcium pantothenate 4 mg l^{-1}, nicotinic acid 20 µg l^{-1}, niacin 4 mg l^{-1}, p-aminobenzoic acid 2 mg l^{-1}, riboflavin 2 mg l^{-1}, thiamine hydrochloride 4 mg l^{-1}.

Inositol 20 mg l^{-1} may be added for growth of inositol requiring mutants.

Difco Agar, 2 % (w/v, added for solid media).

Liquid media was routinely dispensed into conical flasks (100 ml in a 250 ml flask, 400 ml in a 1 l flask) and sterilised by autoclave (15 min at 121 ^{o}C) prior to use. Cells were grown at 25 ^{o}C or 30 ^{o}C in a shaking water bath (120 RPM) or a constant temperature room.

2.3 Solutions

Buffered Sorbitol

1.2 M Sorbitol

10 mM Magnesium chloride.

20 mM Tris adjusted to pH 7.2 with 1 N hydrochloric acid.

0.1 M Sodium metabisulphate.

Tris\HCl Buffer (Bates and Bower, 1956)

50 ml x 0.1 M Tris (hydroxyethyl) aminoethane.

Adjusted to pH 7 with 0.1 M Hydrochloric acid.

Diluted to 100 ml with water.

SH Buffer

272 mM Sucrose.

10 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid).

Adjusted to pH 7.5 with 1 N hydrochloric acid.

Breakage Buffer

50 mM Phosphate buffer, pH 7.4.

2 mM EDTA.

2 mM 2-mercapto-ethanol.

2.4 Determination of Culture Densities and Budding Index

Two hundred microlitres of the yeast culture was diluted to 50 ml with an ultra-filtered (pore-size 0.2 µm) solution of 0.9 % (w/v) sodium chloride and 0.1 % (w/v) sodium azide having determined the background particle count of the solution. Five readings of cell number were made using an electronic particle counter (Particle Data Inc.) which sampled 200 µl of the diluted culture. A stirring arm was available to avoid settling of dense suspensions.

Growth phase was also monitored by measuring optical density at 600 nm on a SP6-UV spectrophotometer (Pye Unicam) and

comparing readings to a 'dry weight' calibration curve. Samples (1 ml) were harvested by centrifugation (30 s at 11600 x *g*) in a Micro Centaur centrifuge (MSE, Fisons, UK) in pre-weighed Eppendorf tubes and washed in distilled water. These were then placed in a vacuum oven (Townsen and Mercer, UK) and incubated overnight to complete dryness. The tubes were then reweighed and dry weights calculated by difference.

Budding index (BI) was determined by briefly sonicating a culture aliquot and inspecting the cells under a light microscope. At least 200 cells were counted and
BI = (Budded cell count)/(Total cell count).

Generation times for yeast cells proliferating in logarithmic phase in liquid media were calculated as follows.

Temperature

Medium	25 ^{o}C	30 ^{o}C	36 ^{o}C
YEPD	135	90	85
YEPE	160	135	135
Glucose Minimal	150	135	130

Times shown in minutes

2.5 Radioactive Tracers

These were all obtained from Amersham International plc, (Bucks., England) and stored according to the suppliers'

recommendations. Radioactivity concentrations are given in Curies (Ci) to facilitate direct comparison with source papers and information. To convert to SI units, 1.0 μCi = 0.037 MegaBequerels (MBq). The detection of radioactive samples was routinely performed in an LKB Wallac 1217 Rackbeta Liquid Scintillation Counter (Turku, Finland).

Myo-[^{3}H]-Inositol, 80-120 Ci mmol^{-1}

Myo-[2-^{3}H]-Inositol with PT6-271, 17.8 Ci mmol^{-1}

Myo-[U-^{14}C]-inositol, 55 mCi mmol^{-1}

L-myo-[U-^{14}C]-Inositol 1-Phosphate, Ammonium salt, 55 mCi mmol^{-1}

D-myo-[2-^{3}H]-Inositol 1,4-Bisphosphate, Potassium salt, 1.0 Ci mmol^{-1}

D-myo-[^{3}H]-Inositol 1,4,5-Trisphosphate, Potassium salt, 60 Ci mmol^{-1}

Phosphatidyl [2-^{3}H] Inositol-4,5-Bisphosphate, 1.0 Ci mmol^{-1}

2.6 Bligh and Dyer (1959) Extraction Technique

This is essentially a lipid extraction technique (Bligh and Dyer, 1959), but one in which 2 layers are produced; the upper layer contains water soluble metabolites whereas the lower "required" layer contains lipid soluble moieties. Yeast cultures were harvested by centrifugation (MSE Centaur 1 bench centrifuge, 10 min at 3120 x *g*) and washed in distilled water. The pellet was assumed to contain 80 % water. An equal volume of chloroform and two volumes of methanol (both BDH, HPLC grade) were added to the pellet

which was then mixed by vortexing for 2 min. Following centrifugation, the supernatant was removed and the treatment was repeated. Two volumes of chloroform were then added to the pooled supernatants and the mixture vortexed for 30 s. Two volumes of water were then added and the sample was again vortexed for 30 s. Centrifugation (10 min at 3120 x *g)* was then used to separate the extracts into a biphasic solution.

2.7 Production of Dowex Column

A glass Pasteur pipette (Bilbate) was taken and broken approximately 1 inch below the "shoulder". A small amount of glass wool was then rolled into a ball and carefully positioned, using a glass rod or similar device, towards the bottom of the Pasteur pipette. The pipette barrel was flushed through with a few millilitres of water to ensure stability of the plug and Dowex anion exchange resin was introduced.

Dowex slurry AG-1X8 (200-400 mesh, formate form, Bio Rad, Hemel Hempstead, Herts., UK), was prepared by mixing the Dowex anion exchange resin in 1:1 (w/v) ratio with distilled water. The slurry was best made in large volumes, and allowed to 'de-fine', whilst stored in the refridgerator (4 ^{o}C). Once resin and water were mixed, the slurry would settle, leaving tiny filaments *viz* 'fines' suspended in the aqueous phase. The aqueous phase was removed and the slurry resuspended. The more times 'de-fining' was performed, the

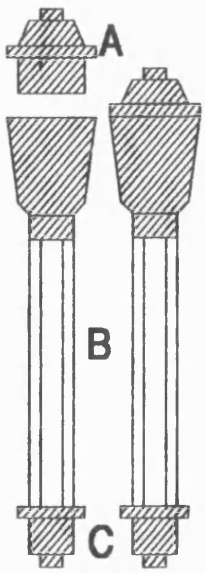
cleaner the Dowex anion exchange resin became, resulting in a faster flow through the column which facilitated separations.

Prior to use, the Dowex anion exchange resin was resuspended by shaking and 1 ml of the slurry was introduced into the Pasteur pipette barrel. Once settled above the glass wool plug, water was flushed through to equilibrate the column, and to ensure that the Dowex resin did not become dry. Pasteur pipettes were replaced during the project by re-usable 0.5 x 10 cm Econo-column chromatography columns (Bio Rad, Herts., UK) which allowed 10 cm Dowex anion exchange columns to be connected to the HPLC apparatus. Dowex anion exchange slurry was poured directly into the column barrel and the resin was equilibrated by flushing with water. Samples of more than 2 ml in volume were frequently analysed using this equipment.

Figure 2.1. represents the Econo-columns. Built in filters allow Dowex resin to be immediately poured in, and connection to HPLC allowed gradients to be run.

Fig. 2.1
Bio Rad
Econo-columns

A: Plastic lid
B: Glass barrel
C: Plastic base with filter



2.8 Elution Regime for Removal of Inositol Phosphates from Dowex Anion Exchange Resin (Nahorski, 1989, Pers. comm.)

The fractions collected from separations by Dowex anion exchange were generally 2 ml in volume, measured by collection of drops into a calibrated disposable plastic micro test tube. Once the sample had been added to the column, and passed completely through, the elution of potentially present inositol phosphates could begin; using a gradient of increasing concentrations of ammonium formate .

Low ionic charge inositol was removed by 7 x 2 ml fractions of distilled water, due to its high solubility. Ammonium formate (5 x 2 ml of 0.025 M) removed glycerophospho-inositol, an unwanted by-product, produced at the cell surface by the deacylation of phosphatidyl-inositol (White *et al.*, 1991). Ammonium formate (7 x 2 ml of 0.2 M in 0.1 M formic acid (BDH)) was used to elute inositol monophosphate (IP_1). Ammonium formate (7 x 2 ml of 0.5 M in 0.1 M formic acid) removed inositol bisphosphates (IP_2) from the column. Inositol trisphosphate (IP_3), the secondary messenger and its isomers were eluted by 5 x 2 ml of 0.7 M ammonium formate in formic acid. Ammonium formate (5 x 2 ml of 1.0 M in 0.1 M formic acid) displaced inositol tetrakisphosphate (IP_4), and finally 5 x 2 ml 2.0 M ammonium formate in formic acid removed any remaining phosphates, such as inositol pentakisphosphate (IP_5) or phytic acid (IP_6).

Dowex anion exchange columns were used only once because at the end of an elution, the resin was recovered from the Pasteur pipette or Econo-column, resuspended in 2 ml water and counted to check that all the radioactivity had been removed. This made it possible to assess the efficiency of radioactivity recovery from the column and monitor tracer distribution throughout an experiment.

2.9 High Performance Liquid Chromatography (HPLC)

Analysis by HPLC was performed using a Partisil (10 micron) SAX 25 cm anion exchange column, coupled with a 2.5 cm guard column packed with pellicular anion exchange resin in an FSA HPLC cartridge system (FSA laboratory supplies, Leics., England).

The high pressure gradient elution systems were provided by 2 Model 305 Piston Pumps (Gilson Medical Electronics Inc, WI, USA), with WSC-type piston pump heads and mobile phase mixing was performed by a Gilson 811B Dynamic Mixer. Internal pressure was monitored by a Gilson 805 Manometric module and fractions were collected using a Gilson FC 203 Fraction Collector (Fig. 2.2)

Samples were injected into a Rheodyne 7125 Loading Sample Injector (Rheodyne, California, USA), using 100 microlitre Hamilton syringes (700 Series), (Hamilton Bonaduz AG, Bonaduz, Switzerland). The elution regime used was from Batty *et al.*, (1985, 1989) after Dean and Moyer (1987). The

eluting gradients were composed of water and ammonium dihydrogen orthophosphate ($(NH_4)H_2PO_4$), adjusted to pH 3.7 using phosphoric acid (H_3PO_4). Following sample injection, free inositol was eluted by washing the column for 15 minutes with water only. The inositol phosphates were then separated by applying 3 consecutive gradients at a flow rate of 1 ml min^{-1}.

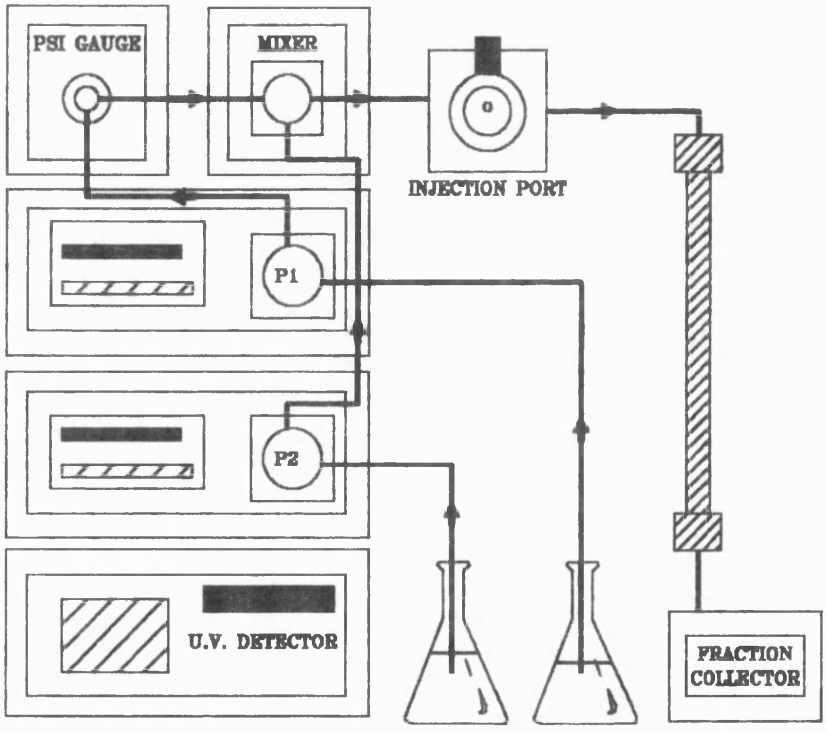


Fig. 2.2. Diagrammatic Representation of the HPLC Apparatus
N.B. P = Pump

A linear gradient of 0-60 mM $(NH_4)H_2PO_4$ was applied over 30 minutes to shift GPI and IP_1. This was followed by isocratic elution at 190 mM $(NH_4)H_2PO_4$ for 15 minutes followed by the

second gradient, a linear increase in $(NH_4)H_2PO_4$ concentration to 300 mM that separated GPIP and IP_2 isomers.

$GPIP_2$ and IP_3 isomers were separated by isocratic elution for 35 minutes at 500 mM $(NH_4)H_2PO_4$. The higher phosphates were eluted from the column by a 15 minute wash with 1.4 M $(NH_4)H_2PO_4$.

In Summary: Solution A = Water
Solution B = $(NH_4)H_2PO_4$ 1.4 Molar
Flow rate = 1 ml min^{-1}

Minutes	Elutant	Comments
00-15	100.0% A	Removed Inositol
15-45	4.3% B	0-60 mM B, Removed IP_1 & GPI
45-46	13.6% B	Shift to 190mM B
46-61	13.6% B	Isocratic 190 mM elution gradient
61-76	21.4% B	to 300 mM. Shifted IP_2 & GPIP
76-77	35.7% B	Shift to 500 mM B then isocratic
77-112	35.7% B	elution to removed IP_3 & $GPIP_2$
112-113	100.0% B	Jump to 1.4 M B
113-128	100.0% B	Isocratic elution of IP_4.

94 ml A, and 34 ml B used.

2.10 Detection of Enzyme Activity

2.10.1 Phospholipase C (Method A)

One hundred millilitres of cells in YEPD were grown to a suitable optical density and harvested by centrifugation. The cell free extract was discarded and the pellet resuspended in 2 ml Breakage buffer or Tris/HCl (See discussion). The cells were broken by shaking with glass beads for 35 seconds in a Braun homogeniser (C. Braun, Melsungen, Germany) and the homogenate was transferred to

Eppendorf tubes through a coarse sieve to remove the glass beads. Centrifugation (1 min at 11600 x *g)* was used to separate the supernatant and pellet. Ten microlitres (0.10 µCi) of PIP_2 was added to the supernatant and this was incubated at 30 ^{o}C for 10 minutes - 2 hours (See Results 3.10). Reactions were terminated by incubation at 100 ^{o}C for 2 minutes. The samples were pelleted by centrifugation and the supernatant was removed and pooled for application to a Dowex anion exchange column. Pellet samples were treated in a similar manner following resuspension in 1 ml of buffer and brief vortex mixing to release water soluble products.

2.10.2 Phospholipase C (Method B)

Mid-exponential cells were harvested by centrifugation and washed in water (10 ml). The cells were then broken by Braun homogenisation in 5-7 ml of the improved buffer (50 mM HEPES, 1 mM $CaCl_2$, 100 mM NaCl, 1 mg^{-1} sodium deoxycholate, 0.5 mg ml^{-1} Bovine serum albumin), and the beads were washed repeatedly until 18 ml of homogenate were recovered. A solution of ^{3}H-PIP_2 was made up in the improved buffer (10 µCi in 2.5 ml) and following the removal of 0.5 ml as a T0 min sample, the substrate and enzyme source were mixed and incubated at 30 ^{o}C for 1 h with shaking (120 RPM). Samples (5 ml) were taken at 5, 20, 40 and 60 min, and reactions were halted by incubation at 100 ^{o}C for 2 min. Supernatants were removed after centrifugation and eluted from 1 ml Dowex resin columns.

2.10.3 IP_3 Phosphatase

Strain MC3 was grown to mid-exponential growth phase and harvested by centrifugation (10 min at 3120 *x g*). The pellet was washed, resuspended in 30 ml of phosphate buffer (pH 7.0) and probe sonicated for 10-15 min. The homogenate was transferred to a sterile 100 ml flask and tracer ^3H-IP_3 (50 µl, 0.5 µCi) was added. The mixture was incubated at 25 ^oC for 60 min during which samples (4 ml) were removed and transferred to sterile Universal bottles. Reaction were halted by boiling for 2 min, and, following centrifugation, the supernatant was applied to a Dowex anion exchange column.

2.11 Detection of Inositol Metabolites

Cells were grown to an appropriate density in 100 ml of defined minimal medium, supplemented with inositol (MM ino^+). Following harvest by centrifugation and disposal of the cell free extract, the cells were resuspended in 100 ml of defined minimal medium without inositol (MM ino^-). The flask was reshaken for two hours at 30 ^oC to starve the cells of inositol.

The cells were reharvested, and the pellet resuspended in 10 ml of MM ino^-. The tracer was then added, usually a known volume of tritiated inositol with or without PT6-271 and equal aliquots of the suspension were pipetted into sterile universal bottles. The tubes were incubated at 30 ^oC for

timed periods before reactions were terminated by boiling for 2 minutes. The solutions were cooled, before centrifugation (10 min at 3120 x *g*) and collection of the supernatants for column introduction.

2.12 Glucose Stimulated Hydrolysis of PIP_2 (Kaibuchi *et al.*, 1986)

Yeast strain MC3 was grown to late exponential growth phase, harvested by centrifugation and washed in distilled water. The pellet was resuspended in MM ino^+ and supplemented with radioactive inositol (20 µCi ml^{-1}). The culture was incubated for 24 hours with shaking at 30 ^oC then reharvested to obtain the primary supernatant. The pellet was resuspended in MES (2-[N-Morpholino]- ethanesulphonic acid)/Tris buffer (0.1 M, pH 6.5) and incubated for 2 hours before stimulation with 25 mM glucose. Incubation continued for 45 min until reactions were terminated and potentially present phosphates were extracted from the secondary supernatant.

2.13 Preparation of Sphaeroplasts

Harvested organisms were washed once in water, then in buffered sorbitol. Cells were suspended in the same buffer to 10 mg dry wt ml^{-1}. Zymolyase 100,000 (Kirin Brewery Co. Ltd., Takasaki, Japan.) was then added (0.1 mg (10 mg dry wt organism)$^{-1}$) and the suspension incubated at 30 ^oC with reciprocal shaking (120 RPM). After incubation for 1 hour, a

check that formation of sphaeroplasts was made by diluting 0.1 ml portions of the suspension into 2.9 ml of both 1.2 M sorbitol and water and measuring the optical density at 600 nm.

2.14 Isolation of Plasma Membranes

Plasma membranes were isolated from sphaeroplasts that had been surface-labelled with cationic silica microbeads (Schmidt *et al.*, 1983; Chaney and Jacobson, 1983). Sphaeroplasts were washed three times (centrifuge at 2000 x *g* for 3 min) in coating buffer (1.2 M sorbitol containing 25 mM sodium acetate and 0.1 M potassium chloride; pH 6.0), the population counted and then suspended in coating buffer to 1.5 x 10^8 sphaeroplasts ml^{-1}. Suspensions of sphaeroplasts and microbeads (3 %, w/v, in coating buffer) were mixed in the ratio 2:1. After incubation for 3 min at 4 ^oC the suspension of coated sphaeroplasts was centrifuged (2000 x *g* for 4 min), the sphaeroplasts washed once in coating buffer and then suspended in the same buffer to a concentration of 10^8 sphaeroplasts ml^{-1}. This suspension was diluted with an equal volume of coating buffer (pH 6.0) containing 2 mg ml^{-1} polyacrylic acid (M_r 90,000) and the sphaeroplasts washed once with coating buffer. The sphaeroplasts were then resuspended in lysis buffer (5 mM Tris-HCl containing 1 mM EGTA; pH 8.0) to 10^8 sphaeroplasts ml^{-1} and the suspension vortexed for 5 min causing at least 95% lysis. The lysate was centrifuged (3000 x *g* for 5 min), the pellet of plasma-membrane washed three times in lysis

buffer and then suspended in 10 ml assay buffer (100 mM MES/Tris, 80 mM KCl, 6 mM $MgCl_2$, pH 6.5).

The protein content of the plasma-membrane preparation was assayed using the Bio-Rad protein assay (Bio Rad Laboratories, Munich, Germany), a method based on the dye-binding technique of Bradford (1976). Portions (0.1 ml) of plasma-membrane preparation were solubilized by boiling for 5 min after addition of an equal volume of 1 M NaOH. The solution was then cooled, neutralized by addition of 1 M HCl (0.1 ml) and 0.2 ml acetic acid-sodium acetate buffer (0.2 M at pH 5.0). The amount of protein in samples was calculated using bovine serum albumin as a standard.

2.15 Lipid Extraction

Pre-washed organisms grown to a suitable phase of growth were mixed with 10-15 ml of ethanol (100 %) at 80 ^{o}C. The mixture was briefly vortexed and incubated at 80 ^{o}C for 15 min in a water bath. This process deactivated lipolytic enzymes and split lipid protein linkages (Letters, 1967). The extract was filtered through a 2.5 cm fine glass microfibre filter (GF/F, Whatman, UK) under vacuum and the filtrate pipetted into a separating funnel. The residue was extracted two or three times with methanol:chloroform (2:1 v/v) for 2 hours as it was stirred magnetically on a flat bed stirrer at room temperature. The extracts were pooled in the separating funnel and washed with a 0.25 volume of

0.88 M KCl according to Folch *et al.* (1957). The mixture was left overnight to separate. The lower organic phase was removed and taken to near dryness in a rotary evaporator prior to further treatment. The upper aqueous layer was often collected, concentrated by incubation in a vacuum oven and analysed by columnn chromatography.

2.16 Mild Alkaline Deacylation of Phosphatides and Glycolipids

Lipid samples thought to contain radioactive phosphoinositides (prepared as described in Materials and Methods 2.15) were transferred to a 15 ml glass-stoppered tube and evaporated to dryness under a stream of nitrogen. To the residue, 0.2 ml chloroform, 0.3 ml of methanol and 0.5 ml methanolic NaOH (0.2 N, freshly prepared and clarified by centrifugation if necessary) were added in succession. The solution was vortexed briefly and left to stand at room temperature for 15 min. Immediately, 0.2 ml methanol, 0.8 ml chloroform and 0.9 ml water were added sequentially and the mixture was again vortex mixed. The sample was then centrifuged for 1 min at 1000 x *g*, to produce a biphasic solution. The upper methanol-water phase was immediately transferred *via* Pasteur pipette to a 15 ml centrifuge tube containing 0.3-0.5 ml of cation exchange resin (Dowex-50 (H^+) cation exchange resin was washed once with 1 N HCl and then with water until the supernatant was neutral. The resin was then drained and used while moist). The mixture was vortexed vigorously until the supernatant

was neutral or slightly acidic to indicator paper (*ca.* pH 5-6). The mixture was again centrifuged and the supernatant transferred to another 15 ml tube. The remaining chloroform layer was washed twice with 0.5 ml of methanol-water (10:9 v/v) and the methanol-water washings were used to wash the ion-exchange resin. The combined methanol-water phases were neutralized with a few drops of methanolic NH_4OH (1.5 N) prior to examination by HPLC.

Method obtained from Kates (1972) after Brockerhoff (1963), Dawson *et al.* (1962), Dawson (1967) and Hübscher *et al.* (1960).

2.17 HPLC Elution of Deacylated Phosphatides

The deacylated products were analysed on the previously described HPLC apparatus using a procedure described by Seyfred and Wells (1984) and Wells *et al.* (1987) after Dittmer and Wells (1969). A polyphasic gradient beginning with 100 mM ammonium formate, pH 9.5 and ending with 750 mM ammonium formate, pH 9.5 was used to elute the phospholipids. Fractions (0.8 ml) were collected at a flow rate of 0.6 ml min^{-1} into scintillation vials. Four millilitres of Optiphase 'Safe' scintillation fluid (LKB, FSA, Loughborough, UK) was added to the samples which were counted in a liquid scintillation counter.

A linear gradient of 0-100 mM ammonium formate was applied over 15 min to remove unwanted compounds of low ionic

strength. This was followed by a second linear gradient to 450 mM ammonium formate (5 min) which eluted GPI from the column. A third linear gradient to 500 mM ammonium formate was applied over 15 min to remove deacylated phospholipid products that were not detectable using a radioactive inositol labelling scheme. A final linear increase in ammonium formate concentration to 750 mM (20 min) eluted GPIP from the column and isocratic elution at this concentration removed $GPIP_2$ and cleared the column.

In Summary: Solution A = Water
Solution B = 750 mM Ammonium formate

Flow Rate = 0.6 ml min^{-1}
Sample size = 1.3 min $fraction^{-1}$

Minutes	Elutant	Comments
00-15.6	13.3% B	Gentle gradient to 100 mM B
15.6-20.0	60.0% B	Gradient to 450 mM B. Elutes GPI
20.0-45.5	66.6% B	Increase to 500 mM B
45.5-65.0	100.0% B	Final gradient to 750 mM B
65.0-78.0	100.0% B	Isocratic elution to clear column

19 ml A, and 28 ml B used.

2.18 Hanson (1991) Style Enzyme Preparation

Organisms grown to a suitable optical density were harvested and washed in water. The pellet was resuspended in approximately 10 ml of a buffer appropriate to the required enzyme activity and this was added to a stoppered Braun bottle containing 30 g of glass beads (Sigma type V; 0.45-0.5 mm diameter). The suspension was shaken in a Braun homogeniser (C. Braun, Melsungen, Germany) for 6 x 15 s periods at 4000 RPM. The samples were cooled continuously by

expanding carbon dioxide passing through the homogeniser. The homogenate was transferred to a centrifuge tube and centrifuged for 5 min at 3000 x *g* to remove the bulk solids. The supernatant was transferred to a second tube and centrifuged at 40000 x *g* for 30 min (RC5C Refrigerated Superspeed Centrifuge, Sorvall Instruments, Du Pont, USA). The pellet was resuspended in a small volume of appropriate assay buffer.

2.19 Enzyme Assay Procedure

The enzyme preparation was suspended in 1.2 ml of assay buffer and vortexed to homogeneity. The radioactive tracer under examination was dissolved in 400 µl of the same buffer. At time zero (T0 min), a 100 µl sample of tracer solution was removed, treated with an equal volume of ice-cold pyridine solution (ethyl alcohol:diethyl ether:pyridine, 15:5:1, v/v/v) after Hanson (1991) and made up to 2 ml with water. The remaining 300 µl of tracer solution was added to the enzyme preparation and the mixture incubated at 25 ^{o}C in a shaking water bath. Samples of 0.5 ml were removed during the course of the incubation and reactions were halted as at T0 min. Following incubation on ice for 10 min, the sample was centrifuged in an Eppendorf tube and the supernatant removed. The pellet was washed twice with 0.5 ml water and the pooled extract was analysed by HPLC. Volumes were altered if extra samples were required, these are described in the text.

The turnover of inositol phosphates was frequently measured by measuring the area-under-the-curve of the chromatograms produced, as apposed to peak-height ratios. The separate peaks were cut out and weighed on a sensitive balance, and directly compared in arbitrary units. To standardize repeat experiments, the weights were converted to percentage values (the T0 min peak value was equivalent to 100%) and evaluated in the same way.

2.20 Gas Chromatography (GC) Analysis of Inositol

HPLC fractions believed to contain inositol were collected in Bijoux bottles and evaporated to dryness in a vacuum oven at 60 ^{o}C. The extract was converted to its oxime by addition of 0.5 ml STOX reagent (Pierce Chemical Co., The Netherlands) and heating to 70 ^{o}C for 30 min. Following cooling the oxime was silylated by adding 0.5 ml trimethylsilylimidazole (TMS) and shaken for a period of 30 s. This was then allowed to stand at room temperature for 30 min. GC analysis was performed using a Pye Unicam PU 4500 capillary GLC. A SE30 column (25 m length; internal diameter 0.22 mm; SGE) was maintained at 200 ^{o}C for 2 min, raised to 300 ^{o}C at 16 ^{o}C min^{-1} and maintained at that temperature for 3 min. Injection and detector temperatures were set at 250 ^{o}C and 350 ^{o}C respectively, with helium as the carrier gas (1 cm^{3} min^{-1}).

2.21 Uptake of PIP_2 using Electroporation

Electroporation was performed with a Gene Pulser Transfection Apparatus (Bio Rad, Herts., UK) which produced high-voltage, high current exponential pulses of controlled characteristics. The gene pulser was attached to a chamber which enclosed and held a sample cuvette between two electrodes.

Harvested cultures were washed once in SH buffer (272 mM sucrose, 10 mM HEPES) pH 7.5 then resuspended in 6-10 ml SH buffer pH 7.4 after Hashimoto *et al.* (1985). Samples (1 ml) were transferred to sterile disposable gene-pulser cuvettes (Bio Rad) with an electrode gap of 0.4 cm. Following addition of radioactive tracer PIP_2 the cuvettes were pulsed at the capacitance and voltages described later.

Samples that required incubation were placed in a shaking water bath at 25 ^{o}C. Extraction of putative inositol phosphates was performed by transferring the cuvette contents to an Eppendorf tube and harvesting by centrifugation (1 min at 11600 x *g*). The pellet was then washed in water and subjected to the Bligh and Dyer extraction procedure described in 2.6. Extracts were examined by column chromatography.

To monitor the efficiency of electroporation a pulsed sample was serially diluted in 25 % Ringers solution. Samples (100 µl) of 10^{-4}-10^{-6} dilutions were plated out in duplicate on

YEPD and incubated at 30 ^{o}C for 2 days. Viability was calculated by comparison with a non-pulsed sample.

2.22 Thin Layer Chromatography (TLC)

All separations were performed using flexible polyester-backed TLC plates coated with silica gel 60A (250 µM, Whatman, UK) and pretreated by impregnation with potassium oxalate. Plates were developed in a methanol:water (2:3 v/v) mixture containing 1 % potassium oxalate, then left to dry at room temperature (Jolles *et al.*, 1981).

Extracted and filtered lipids were reduced under nitrogen and applied to the plate by repeated spotting with a micro-pipette. Samples were run alongside 2-6 µl spots of genuine phospholipid standards including *bona fide* phosphoinositides.

Plates were developed in Shandon chromatography tanks that were lined with filter paper and allowed to equilibrate for at least 3 hours at room temperature. The mobile phase originally consisted of chloroform: methanol: ammonium hydroxide (45:35:10 v/v/v) after Billah and Lapetina (1982) but was altered to chloroform: acetone: methanol:glacial acetic acid:water (40:15:13:12:8 v/v/v/v/v) after Jolles *et al.* (1981).

The plate was removed from the tank and allowed to dry in a

fume cupboard when the solvent front had ascended approximately 75-80% of the plate. Lipid separations were visualised by placing the plate in a tank containing iodine vapour, and samples were identified on the basis of similar Rf values to those of *bona fide* materials.

Rf = Distance travelled by sample / Distance travelled by solvent front

Bands believed to contain radioactivity were cut from the plate and placed in vials with Optiphase 'Safe' (5 ml) for detection by scintillation counting.

3.0 Results

3.1 Elution of Standards

For the accurate identification of an unknown compound eluted from any ion exchange column, it was essential to chart the elution of known standards. This allowed retention properties to be characterised and elution regimes to be confirmed.

3.1.1 Inositol

Following storage of myo-[^{3}H]-inositol for several weeks, a sample was analysed by elution from both Dowex and HPLC anion exchange columns. However the expected rectangular hyperbola or exponential-like 'decay', with 95% of the activity removed by flushing through with water was not seen. As opposed to the asymptotic approach to zero counts, several peaks were present particularly in the 'IP_1' and 'IP_2' windows, neither of which should have been present (Fig. 3.1). Tritiated inositol decomposes with time (S.Qazi, Pers. Comm.; Amersham Catalogue, 1990) producing radiolytic decomposition products. The impurities are known to co-elute with myo-inositol phosphates (using a Dowex AG1-X8 column) using the elutants that remove IP_1 and IP_2. Tritiated inositol was purchased containing PT6-271, a novel polymer that absorbs the polar impurities of decaying myo-inositol. This should remove breakdown products, ensuring high purity and low blanks. However impurity peaks were again discovered

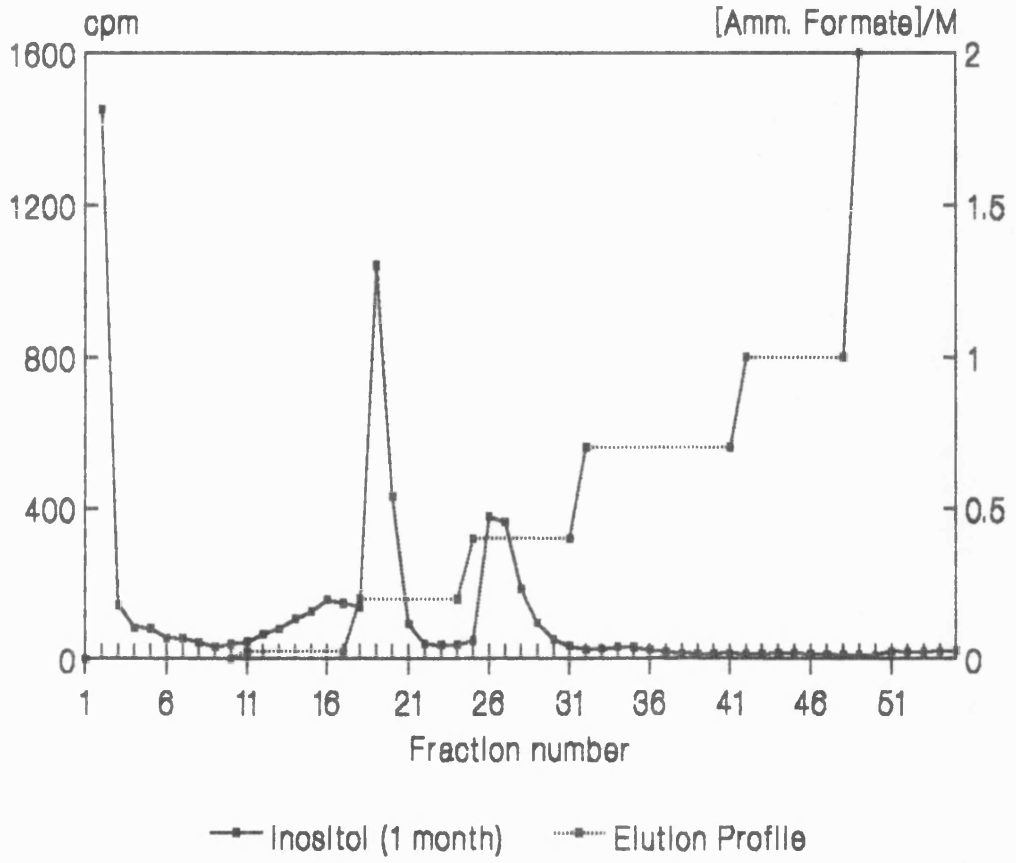


Fig. 3.1 Elution of Tritiated Myo-Inositol

One microlitre (1.0 µCi) of myo-[^{3}H]-inositol in 1 ml distiled water was applied to a 1 ml Dowex anion exchange column (formate form) and eluted stepwise with solutions containing increasing concentrations of ammonium formate /formic acid (0.1 M), having been stored at 4 ^{o}C for 1 month. Fractions (2 ml) were collected and mixed with 8 ml Optiphase 'Safe' for detection by liquid scintillation counting.

after a period of storage. ^{14}C-labelled inositol was bought as a more stable tracer and had not developed contaminant peaks after six months storage (Fig. 3.2).

3.1.2 Inositol Trisphosphate

Due to the nature of the tritium label, stored ^{3}H-IP_3 also produced unexpected peaks in its elution profile. IP_3 degrades with time to produce radioactive IP_2 and IP_1 (Fig. 3.3) which elute with the correct retention properties of these inositol phosphates. The contaminating species could be accounted for if a sample column was run as an external standard.

Analysis by Amersham plc (UK) has indicated that the peak in the bisphosphate position is composed of a mixture of both the 1,4 and 1,5 isomers, but it is not known whether the peak in the monophosphate position is composed of the 1, 4 or 5 analogue or is indeed a mixture of all three (Webb, A., 1991; pers. comm.).

Experiments to determine detection levels have been performed by HPLC. As single samples, IP_3 is detectable down to 8 fM and IP_1 to 0.6 pM. IP_2 has been detected down to a concentration of 0.53 pM and in experiments to monitor the possibility of large IP_2 peaks masking IP_3, an IP_3 peak was still detectable when IP_2 was present in a peak height ratio

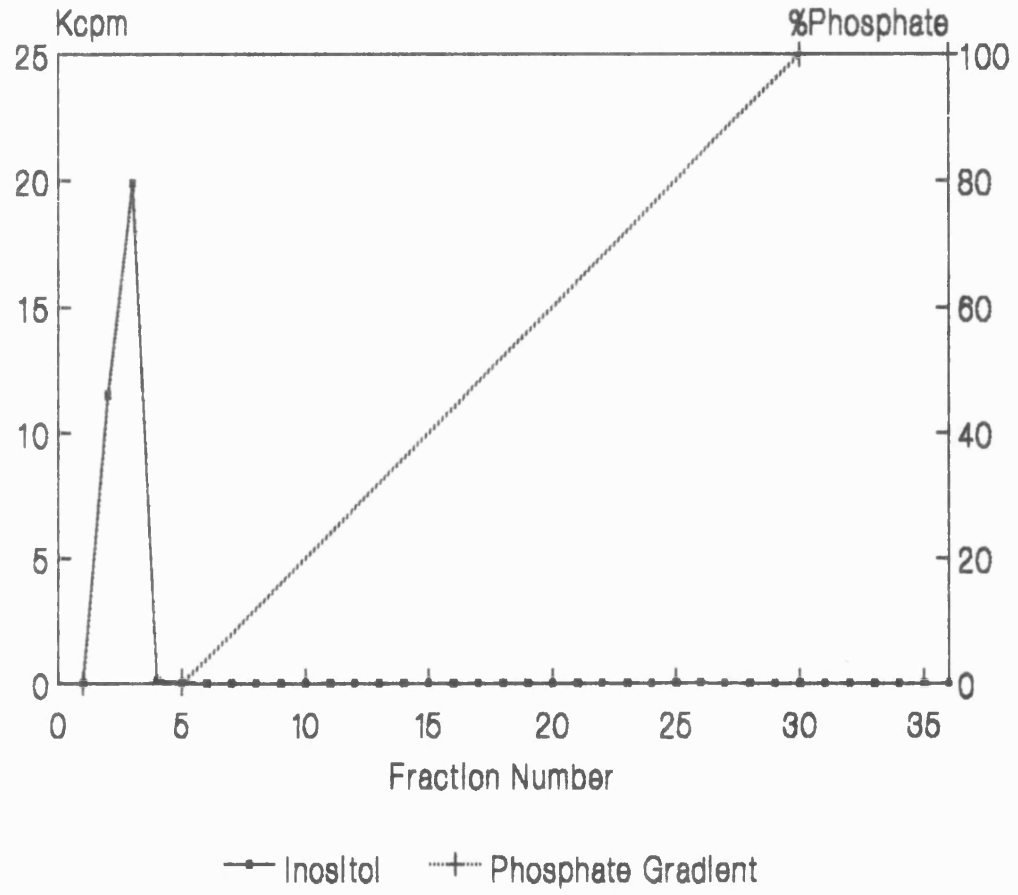


Fig. 3.2 Elution of Carbon Labelled Inositol

Myo-[U-^{14}C] inositol, 0.5 µl (0.013 µCi) in 20 µl was eluted from a 10 µm Partisil SAX HPLC column with a linear gradient (0-100% $NH_4H_2PO_4$, adjusted to pH 3.7 with H_3PO_4). Flow rate was 1 ml min^{-1} at ambient temperature. All counts were successfully removed from the column during the initial water wash and no contaminant peaks were detected. Fractions (2 ml) were collected and mixed with 8 ml Optiphase 'Safe' for detection by liquid scintillation counting.

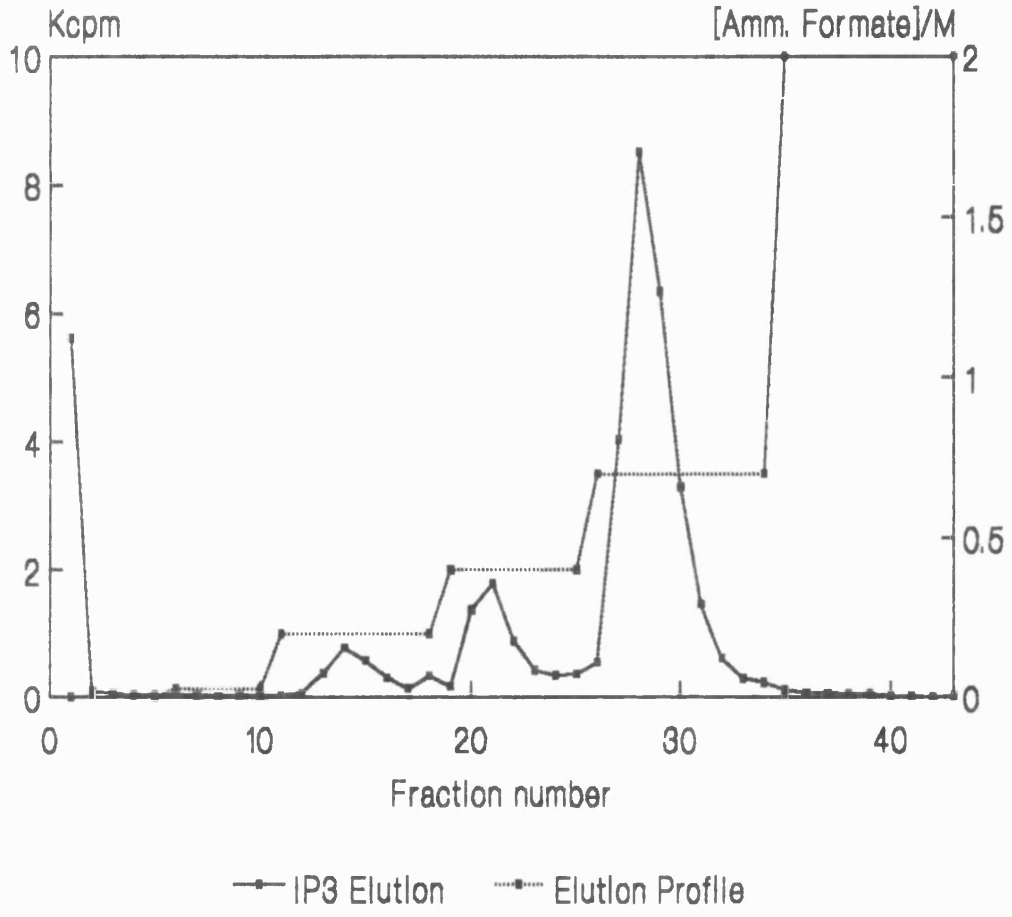


Fig. 3.3 Elution of Tritiated IP_3

Twenty five microlitres (0.25 µCi) of D-myo-[^{3}H]-inositol 1,4,5-trisphosphate (potassium salt) in 1 ml distilled water was eluted from a 1 ml Dowex anion exchange column using a step-wise elution system of increasing concentrations of ammonium formate. Apart from the expected main peak in the IP_3 window, secondary peaks were also detected in the IP_2 and IP_1 windows.

of 50:1. At a peak ratio of 75:1 (IP_2:IP_3), the IP_3 peak was almost incorporated in the baseline and could not be distinguished at a ratio of 100:1. No masking was observed from peak shoulders or co-elution of standards.

3.1.3 Inositol Bisphosphate

Tritiated IP_2 (D-myo-[2-^{3}H]-inositol 1,4-bisphosphate) was eluted from an HPLC column at the expected retention time for a linear gradient (Fig. 3.4). Some degradation of the main peak was observed with time, the increase in radioactivity occurring at the IP_1 position. It is not known which isomer of IP_1 was produced by the tritium decay. The linear gradient used for the majority of analyses did not distinguish between isomers, so it was not known if the contaminant peak contained Ins(1)P_1, Ins(4)P_1 or a mixture of both.

3.1.4 Inositol Monophosphate

[U-^{14}C]-labelled IP_1 has been successfully eluted from both HPLC (Fig. 3.5) and Dowex anion exchange chromatography columns in the correct position with no contaminants.

Higher inositol phosphates were not purchased; the identification of IP_4 isomers, or further phosphorylated intermediates was based on the appearance of novel peaks in expected retention positions according to established elution regimes.

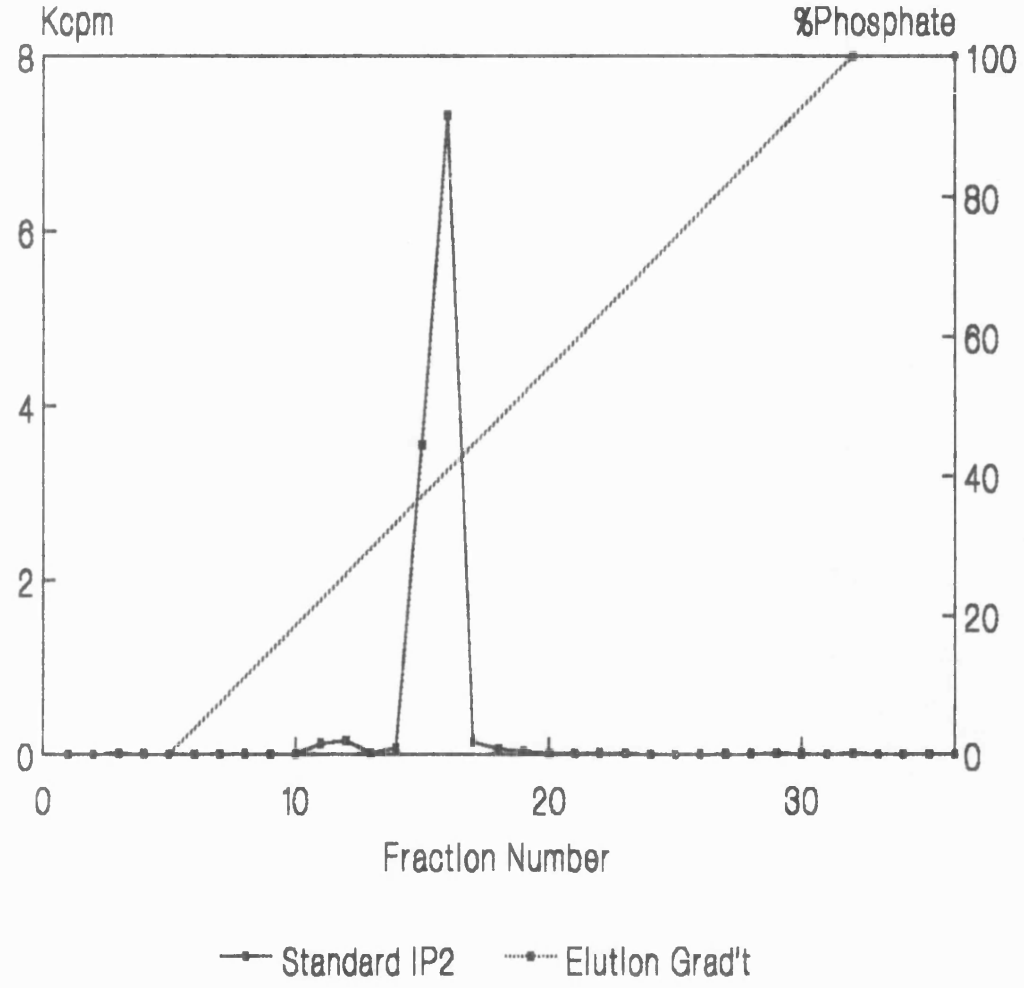


Fig. 3.4 Elution of Tritiated Inositol Bisphosphate

D-myo-[2-^{3}H]-Inositol 1,4-Bisphosphate (1 µl,potassium salt, 0.01 µCi) was eluted from a 10 µm Partisil SAX HPLC column using a linear gradient of $NH_4H_2PO_4$ adjusted to pH 3.7 with H_3PO_4. Fractions (2 ml) were collected at a flow rate of 1 ml min^{-1} and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation counting.

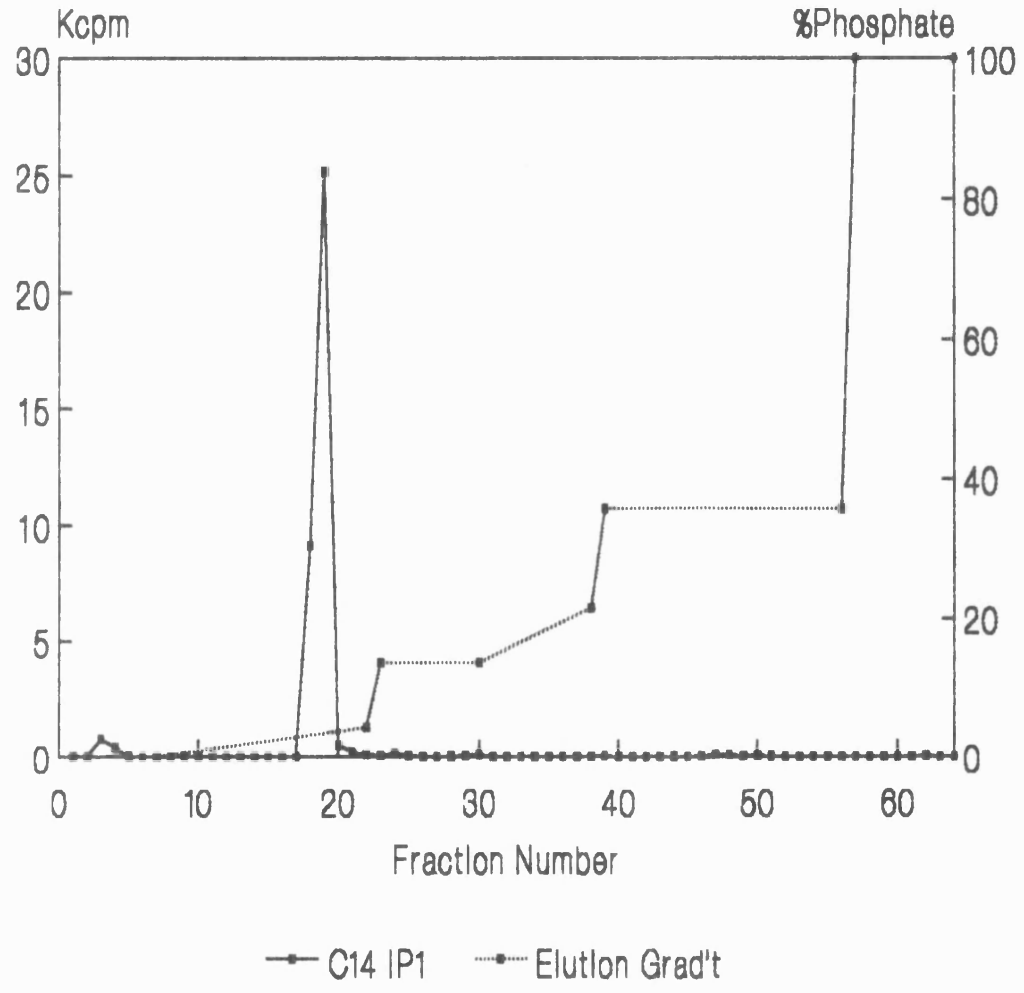


Fig. 3.5 Elution of Carbon Labelled Inositol Monophosphate

L-myo-[U-^{14}C] inositol-1-phosphate (ammonium salt, 0.5 µl, 0.013 µCi) was eluted from an Partisil 10 µm SAX HPLC column using the elution regime of Batty *et al.*, (1985, 1989). Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for liquid scintillation counting. No impurity peaks were detected throughout the elution.

3.2 Distribution of Activity

A principal factor in the development of an inositol phosphate assay is knowledge of the efficiency by which added radioactive tracer is extracted or partitioned at each stage of treatment. *Saccharomyces cerevisiae* has an active uptake system for inositol (Henry, 1982; Nikawa *et al.*, 1982) but early experiments showed that only a very low percentage of added ^{3}H-inositol radioactivity was recoverable from a harvested cell pellet. Activity distribution tests in YEPD showed that up to 60% of the original radioactivity could be lost overnight from a non-inoculated flask (data not shown). This was not seen in parallel flasks of water or minimal medium (MM). Further investigation indicated that the medium components of yeast extract (YE) and peptone (P) together were responsible for activity loss interactions. Theories of heavy compounds or adherence to the glass vessel were refuted. Perhaps YE contained inositol degrading enzymes which allowed tritium to escape into the atmosphere as water vapour.

Although ^{3}H-inositol in uninoculated MM displayed no loss of radioactivity with time, it was found to lose radioactivity at a similar rate to YEPD if inoculated with yeast cells. The distribution of activity experiment described below failed to produce a balanced "account sheet" of radioactivity throughout the extraction process.

Flasks (3 x 200 ml) containing 30 ml MM, were inoculated with strain A364A. An equal quantity (1 µl, 1.0 µCi) of myo-[^{3}H]-inositol tracer was added to each flask and zero time samples were taken to calculate total activity. The flasks were then incubated until the cells had achieved a suitable growth phase.

The cells were harvested by centrifugation and the pellets washed in distilled water. Samples of the growth medium supernatants were counted and the pellet from Flask 1 was resuspended in water (2 ml) for counting.

The pellets from Flasks 2 and 3 were treated with 2 ml of 70% (v/v) perchloric acid (PCA), incubated on ice for 30 minutes then centrifuged to obtain 2 fractions, soluble and insoluble. The soluble supernatants were collected and the radioactivity within it was counted. The pellet from flask 2 was resuspended in 2 ml of water and counted for radioactivity content.

The contents of flask 3 were treated identically to flask 2 except that the pellet was resuspended in 3 ml of extraction fluid (methanol:chloroform 2:1 (v/v)), and placed in a shaking incubator for 10 to 25 minutes at 25 ^{o}C. The sample was then centrifuged, and the soluble fraction collected and counted for radioactivity content. The pellet was re-extracted, and the extract collected and counted by liquid scintillation spectrometry. Any remaining pellet material

was resuspended in 2 ml of water for counting. Results are summarised in Table 3.1.

One hundred percent recovery of radioactivity was finally achieved however by placing the flasks containing radioactivity on ice for 30 minutes post-incubation to recondense any tritiated water vapour. Also PCA treatment was replaced by TCA, as it was thought that PCA might exacerbate loss of radioactivity. It failed to yield adequate pellets after centrifugation and it was difficult to recover all the cells.

3.3 Investigation of Extraction Techniques

Experiments performed throughout the course of the project had shown that in many cases, although radioactivity could be recovered from inactivated cells, a great deal of activity remained within the pellet material. As soon as the pellet was subjected to an extraction process however it became impossible to retrieve the same amount of radioactivity as measured in the intact pellet. A number of extraction procedures were therefore investigated to maximise radioactivity recovery from the pellet.

Cultures of yeast were grown to late exponential stage in YEPD with myo-[^{3}H]-inositol tracer. Following recondensation of any tritiated water vapour, the cultures were harvested by centrifugation and the cell free extract (CFE) sampled for radioactivity content. The pellet was treated with one

Table 3.1 Distribution of Radioactivity Counts During Extraction in Minimal Medium

Time 0 Total Radioactivity (cpm). Flask 1 = 384294
Flask 2 = 389217
Flask 3 = 368202

Flasks were inoculated and incubated at 30°C for 24 hours.

Post-harvest supernatant of Flask 1 contained 102059.1 cpm
" " " Flask 2 " 101150.3 cpm
" " " Flask 3 " 100036.5 cpm

Flask 1 pellet (in 2ml of water) contained 79551.0 cpm

Therefore, total recovery from Flask 1 = 181610.1 cpm

This corresponded to 47.26% of the original radioactivity

The pellets from Flask 2 and 3 were incubated on ice in 2 ml of 70% (v/v) PCA, then centrifuged to separate soluble and insoluble phases.

Flask 2 soluble phase contained 280.6 cpm

Flask 3 soluble phase contained 305.8 cpm

Flask 2 insoluble phase + washings contained 27849.0 cpm

Therefore total recovery from Flask 2 = 28129.6 cpm

This equated to 35.4% of the Flask 1 pellet count.

This corresponded to 7.23% of the original radioactivity

The pellet of Flask 3 was incubated for 25 min at 30 °C in 3ml MeOH:$CHCL_3$ (2:1, v/v), then centrifuged to separate soluble and insoluble phases.

Soluble phase contained 607.4 cpm

Insoluble phase contained 1834.8 cpm

Flask 3 Total Recovery = 2442.2 cpm

This corresponded to 0.66% of the original radioactivity

of a number of extraction techniques. A TCA extraction was performed with and without glass beads (1) after Berridge *et al.*, (1983) and a comparison was made between a normal Bligh and Dyer (1959) extraction and a similar method involving glass beads (2) (Holland *et al.*, 1988; Auger *et al.*, 1989a)

(Method 1). TCA Extraction with and without glass beads. The pellet was suspended in 2 ml of 8% TCA (w/v), vortexed and incubated on ice for 1 hour. Glass beads (0.3 g ml^{-1}) could be included with intermittent vortex mixing. The mixture was then centrifuged and the supernatant collected for further analysis.

(Method 2). Methanol:Chloroform Extraction with and without glass beads. The pellet was treated with 3 volumes of MeOH:$CHCl_3$ (2:1 v/v). Glass beads (0.3 g ml^{-1}) could be added at this point and the mixture vortexed intermittently for one hour at room temperature. The extraction was completed by the addition of 1 volume $CHCl_3$, then 1 volume of water with accompanying vortex mixing. Brief centrifugation produced two layers from which the upper aqueous methanolic phase containing the radioactivity could be removed.

The results (Table 3.2) showed none of the methods to be particularly satisfactory even though the actual uptake of radioactivity was quite low. The total recovery of radioactivity using these techniques reached only 80%, the majority of which was detected in the CFE, and up to 18.9%

of the radioactivity added could remain within the pellet material. These methods were not considered exhaustive enough for further use.

Cell permeabilization techniques, to puncture or fracture the yeast cell wall were therefore considered in an attempt to augment the recovery of soluble phosphates (Methods 3-5).

(Method 3). Potassium phosphate buffer/isopropanol (Srienc *et al.*, 1983). The pellet was resuspended in 5 ml potassium phosphate buffer (0.02 M, pH 7.0). Isopropanol (2.5 ml at 0 ^{o}C) was then added and the solution incubated on ice for 10 minutes. The cells were washed in ice cold water before an extraction was performed.

(Method 4). 2-mercapto-ethanol (2ME). The pellet was resuspended in 2 ml of 0.1M 2ME and incubated for 1 hour at 30 ^{o}C. Cells were washed in distilled water prior to extraction.

(Method 5). Hot ethanol (Rose, A.H., 1990; pers. comm.). Ethanol (2 ml of 70% v/v) at 80 ^{o}C was added to the pellet. Samples were incubated at 80 ^{o}C for 15 minutes, cooled and then centrifuged. The supernatant was removed prior to extraction.

Of the methods used, the phosphate/isopropanol treatment and 2-mercapto-ethanol (2ME) were found unsatisfactory. These methods were less efficient at recovering radioactivity than

those previously tried and cleaning the sample post treatment was considered problematic. It was thought that soluble compounds might have been lost in the washes prior to the Bligh and Dyer (1959) treatment. The hot ethanol extraction was more exhaustive both in terms of extraction from the pellet and total recovery. Up to 90% of the added radioactivity could be recovered, and the ethanol extract itself contained up to 24% of this total - at least three times more than any other method. It was noteworthy that almost negligible counts were recovered *via* a Bligh and Dyer (1959) extraction implying an effective single-step recovery method. Results are summarized in Table 3.2. This technique was not adopted immediately as a change of approach was introduced, but the experiments were important in developing an exhaustive extraction from intact cells.

3.4 Elution of Inositol Phosphates from Dowex Anion Exchange Resin

Using a phosphate elution regime (Nahorski, R.H., 1989; pers. comm.; Ellis *et al.*, 1963). *Sacch. cerevisiae* cells were examined for incorporation, extraction and assay of a radioactive substrate.

Primarily, it was considered important to demonstrate that when dealing with dead or inactive cells, it was possible to recover at least 90% of any added radioactivity. If radioactivity could not be effectively recovered from cells which could not metabolize or take up the tracer, then it

Table 3.2

Recovery of Tritiated Inositol Tracer Using Different Extraction Techniques

1. Methanol:chloroform with and without glass beads

	'Without'	'With'
Mean total recovery	69.16 +/- 5.07%	79.70 +/- 1.92%
Counts in CFE	64.55 +/- 3.76%	72.69 +/- 1.34%
Post B&D aqueous phase	0.69 +/- 0.01%	1.86 +/- 1.10%
Bead wash count	NA	2.53 +/- 0.52%
Solid remains	3.96 +/- 1.28%	3.54 +/- 1.63%

2. TCA treatment with and without glass beads

	'Without'	'With'
Mean total recovery	77.33 +/- 2.64%	76.07 +/- 0.11%
Counts in CFE	56.44 +/- 2.16%	58.23 +/- 2.98%
Post TCA supernatant	2.11 +/- 0.37%	4.47 +/- 0.51%
Bead wash count	NA	1.14 +/- 0.10%
Solid remains	18.79 +/- 0.11%	12.23 +/- 3.28%

3.Isopropanol/phosphate buffer

Mean total recovery	63.16 +/- 1.89%
Counts in CFE	58.19 +/- 1.44%
Post buffer supernatant	2.08 +/- 0.14%
Post B&D aqueous phase	0.70 +/- 0.30%
Solid remains	0.88 +/- 0.13%

4. 2-mercapto-ethanol (2ME)

Mean total recovery	74.77 +/- 3.22%
Counts in CFE	55.44 +/- 2.41%
Post BME supernatant	7.72 +/- 0.50%
Post wash supernatant	6.86 +/- 4.54%
Solid remains	1.60 +/- 1.32%

5. Hot ethanol

Mean total recovery	89.48 +/- 5.89%
Counts in CFE	55.44 +/- 5.19
Post EtOH incubation	16.78 +/- 6.39%
Post B&D aqueous phase	1.86 +/- 0.96%
Solid remains	0.75 +/- 0.41%

6. Hot ethanol repeat

Mean total recovery	89.53 +/- 1.42%
Counts in CFE	65.18 +/- 0.61%
Post EtOH incubation	23.62 +/- 1.68%
Post B&D aqueous phase	0.33 +/- 0.07%
Solid remains	0.33 +/- 0.13%

CFE = Cell Free Extract
B&D = Bligh and Dyer extraction
Etoh = Ethanol
NA = Not Applicable

was not plausible to expect to recover it from growing, viable cells.

A series of experiments were done, demonstrating that a satisfactory percentage of radioactivity could be recovered (Table 3.3). Washed cells were sequentially suspended in reaction-stopping solutions then extractions were performed to collect any radioactivity which might have penetrated the cell. All fractions were collected and counted for radioactivity content.

The recovery of tritiated inositol was judged to be satisfactory because 101.1% recovery of the radioactivity was recorded if the extraction procedure only involved 2 water washes (Table 3.4).

YEPD containing tritiated inositol was inoculated and incubated overnight. A number of methods were employed to periodically halt cell growth and metabolism. It was important to ensure that all enzyme activity had been stopped, preventing possible degradation of higher phosphates back to inositol. Following harvesting of the cells by centrifugation, the pellet was resuspended in 8% (w/v) Trichloroacetic acid (TCA) and left on ice for 10 min. The Bligh and Dyer (1959) extraction technique was used to partition any labelled inositol phosphates. Elution of both aqueous and organic layers from a Dowex anion exchange column showed that the incorporated radioactivity was present only in the upper methanolic phase. Water soluble

Tracer Used	PIP_2	IP_3	Ins
Extraction Step	Percentage Recovery		
40% TCA	50.5	60.3	96.61
10% TCA	12.8	12.86	2.83
1st Extract	12.20	0.4	0.31
2nd Extract	0.69	0.04	----
Solid Remains	23.5	1.55	0.03
Total Recovery	99.69	75.11	99.7

Table 3.3. Recovery of Tracer from Inactivated Yeast Cells

A culture (100 ml) of mid-exponential phase yeast cells (strain MC3) was harvested by centrifugation and washed in water (10-20 ml). The pellet was resuspended in 40% (w/v) TCA to a final concentration of 11% (with respect to TCA) and vortex mixed, the relevant tracer was then introduced and the solution was incubated on ice for 30 min. The supernatant was obtained by centrifugation and counted for radioactivity content. The pellet was resuspended in 2 ml x 10% (w/v) TCA and the procedure repeated. The pellet was then subjected to 2 ethanol:ether (3:1, v/v) extractions. After resuspension the solution was incubated at 60 ^{o}C for 10 min then cooled. Supernatants were obtained by centrifugation and were all counted for radioactivity content and the pellet (in 2 ml of water) was also counted. The results shown are the means of duplicate investigations.

Extraction Step	Percentage Recovery
1st Water supernatant	79.8
2nd Water supernatant	19.6
Solid remains	1.6
Total recovery	101.1

Table 3.4. Recovery of Inositol from Inactivated Yeast Cells Using Water Washes

A culture (100 ml) of mid-exponential phase yeast cells (strain MC3) was harvested by centrifugation and washed in water (10-20 ml). The pellet was resuspended in 40% (w/v) TCA to a final concentration of 11% (with respect to TCA) and incubated on ice for 30 min. Following centrifugation, the supernatant was removed and the pellet was resuspended in 10 ml of distilled water. Tracer myo-[^{3}H]-inositol (1 µCi) was added and the mixture was vortex mixed. Following incubation on ice (30 min), the solution was centrifuged and the supernatant removed for detection of radioactivity content. The pellet was resuspended in distilled water (10 ml) and the process repeated. Results shown are the mean of 2 independent tests.

compounds were detected in the methanolic phase only. The organic layer showed only background counts when analysed by ion exchange chromatography (Fig. 3.6).

Experiments examining the efficiency of a single (Bligh and Dyer only) or double (TCA and Bligh and Dyer) extractions, or the application of cell debris to a column proved inconclusive and uninformative. Although the double extraction appeared to be a more exhaustive method by extracting a greater amount of radioactivity, the peak sizes were not consistently larger than those from the single extraction. Also, experiments performed with increased labelling concentrations suggested that similar radioactivity recovery could be obtained using either method.

Similarly, the application of cell debris to a Dowex anion exchange column appeared to improve radioactivity yield but again the results were not consistent. The elution was much slower than normal and the practice was discontinued because it would have been impossible to incorporate the technique into HPLC analyses.

Following sample application to a Dowex anion exchange column and subsequent washing with distilled water, it was possible to demonstrate that further radioactivity was released when the eluting buffer capable of removing $IP_{5/6}$ was applied (Fig. 3.7).

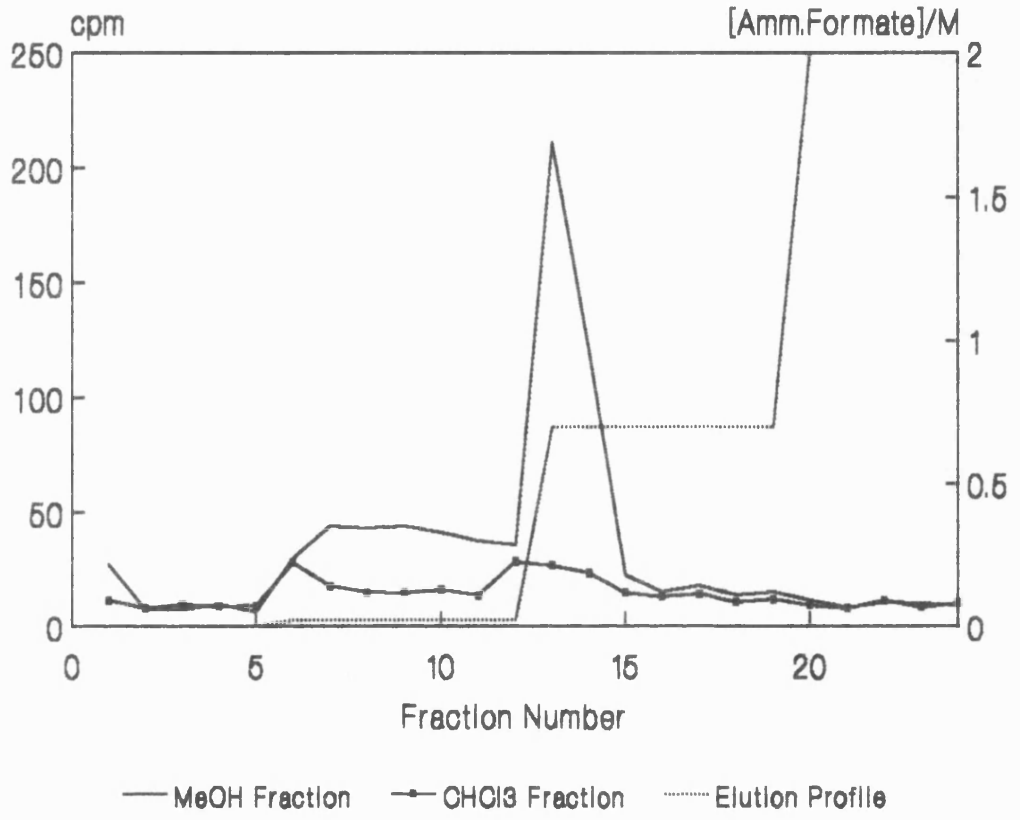


Fig. 3.6. Elution of Aqueous and Organic Layers of a Bligh and Dyer Extraction from Dowex Anion Exchange Resin

Strain A364A was grown to late exponential phase in 100 ml YEPD + 1.0 µCi myo-[^{3}H]-inositol. Following cell harvest by centrifugation, the pellet was washed and labelled compounds extracted using the method of Bligh and Dyer (1959). Each phase was eluted from a 1 ml Dowex resin column with increasing concentrations of ammonium formate/formic acid. Fractions (2 ml) were manually collected and mixed with Optiphase 'Safe' (8 ml) for liquid scintillation counting.

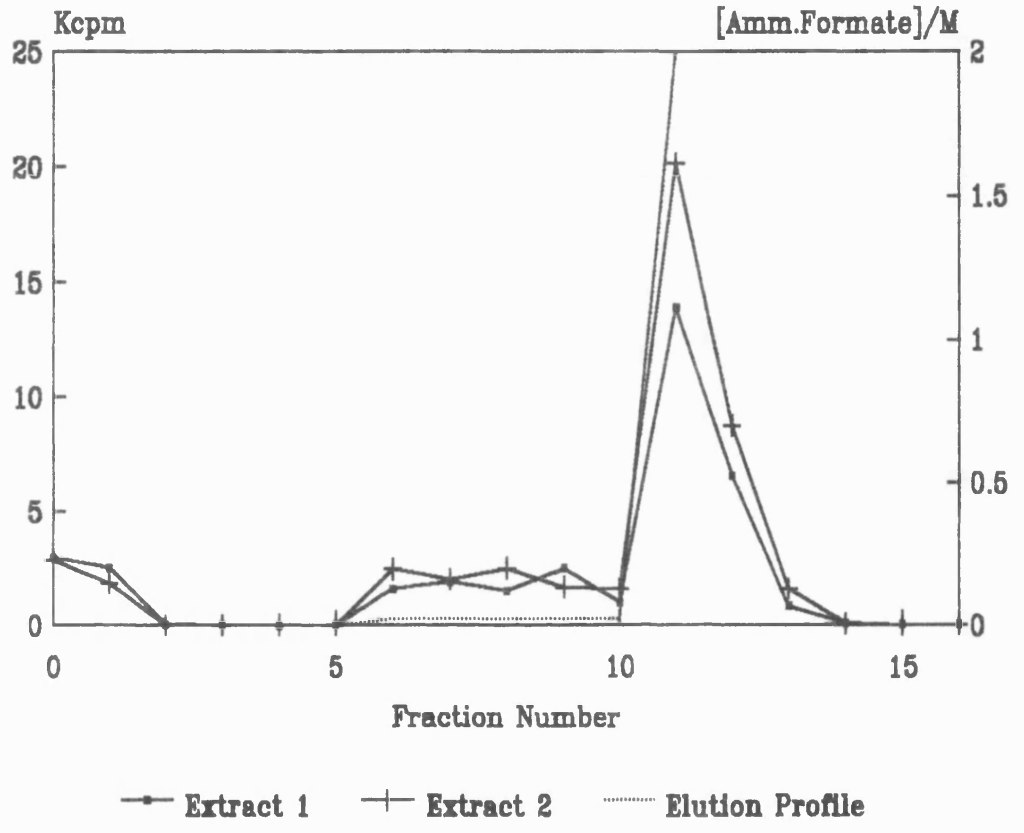


Fig. 3.7. Bulk Removal of Radioactivity from Dowex Resin

Strain A364A was grown to late exponential/stationary phase in YEPD with 10 µCi of myo-[^{3}H]-inositol, then harvested by centrifugation. Radioactivity was extracted from the pellet *via* the Bligh and Dyer (1959) technique and the upper aqueous phase eluted from Dowex anion exchange resin (1 cm). Following the removal of inositol and GPI, the column was eluted with mobile phase strong enough to remove all known inositol phosphates from the resin. Fractions (2 ml) were collected and counted by liquid scintillation spectrometry.

Confident that inositol phosphates were being extracted, a stepwise elution profile was introduced to separate inositol, GPI, IP_3 and any higher phosphates. Any 'IP_3' peak produced was disproportionate however, as a mobile phase capable of eluting IP_3 would also remove IP_1 and IP_2 from the column. It was therefore necessary to include further steps to isolate these intermediates. Although separate peaks were obtained (Fig. 3.8), their identities had not been confirmed. It was not known at this point that they were composed mainly of radiolytic breakdown products. No IP_3 was detected.

Similar results were obtained in cultures grown for only 12 hours, which were in exponential growth phase. This indicated that cells did not have to reach stationary phase for maximal labelling to be achieved, when flux of biochemical pathways may be slowing down or ceasing.

Examining what appeared to be 2 peaks occurring in the IP_2 window, and concerned with the possibility that IP_3 may have been co-eluting with IP_2, the concentration of the elution buffers were broken down into 0.5 M steps as 2 compounds with different ionic charges should not elute at one mobile phase concentration. IP_3 was still not seen in 12 or 24 hour cultures and the dual peaks appeared to be scintillation counting errors. Recounting the same vials failed to produce the same reults - only background radioactivity was observed.

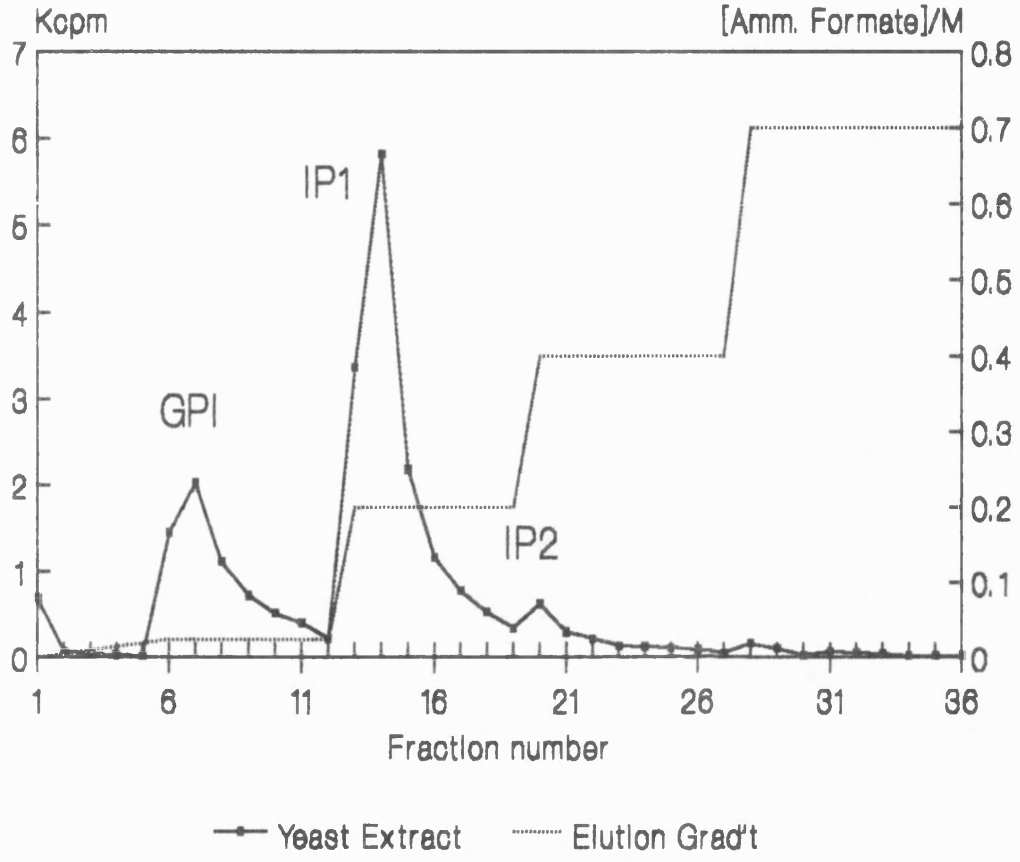


Fig. 3.8 Separation of Lower Inositol Phosphates

A culture of yeast was grown to late exponential phase in the presence of myo-[^{3}H]-inositol tracer. Following harvest of the cells by centrifugation, inositol phosphates were extracted by the method of Bligh and Dyer (1959) and applied to a 1 ml Dowex anion exchange column. Individual inositol phosphates were eluted using a step-wise gradient of increasing ammonium formate concentrations. Fractions (2 ml) were collected and mixed with 8 ml of Optiphase 'Safe' fluid for counting by liquid scintillation spectrometry.

3.5 Detection of Inositol Phosphates

In an attempt to maximise tracer uptake by yeast cells, a protocol was devised in which cells were incubated in defined MM without inositol (MM ino$^-$) to starve them of the substrate before addition of the radioactive tracer (see Methods and Materials 2.11). Following the starvation, strains MC3 and A364A were incubated for timed periods (5-20 min) with tracer inositol (myo-[^{3}H]-inositol, 1.5 µCi). Extracts from the 20 minute samples were eluted from Dowex anion exchange columns. The profiles obtained showed the presence of peaks which exhibited retention properties corresponding to GPI and IP_1. It was considered that these peaks may have been the end products of a degradation pathway, and that samples taken at 5 and 10 minutes may have contained higher inositol phosphates, not yet broken down. The magnitude of these peaks was however seen to increase with time, particularly in strain MC3 (Fig. 3.9) and products such as IP_2 and IP_3 were not detected. Peaks were recorded in the samples from both strains although more radioactivity had been incorporated into the MC3 extracts. It was therefore decided to continue experiments with this strain only. It had been demonstrated that GPI and IP_1 could be isolated from a yeast culture following inositol starvation.

It was further shown that if the period of incubation was increased (30-60 min), then a peak corresponding to IP_2 could also be detected in conjunction with GPI and IP_1 (Fig.

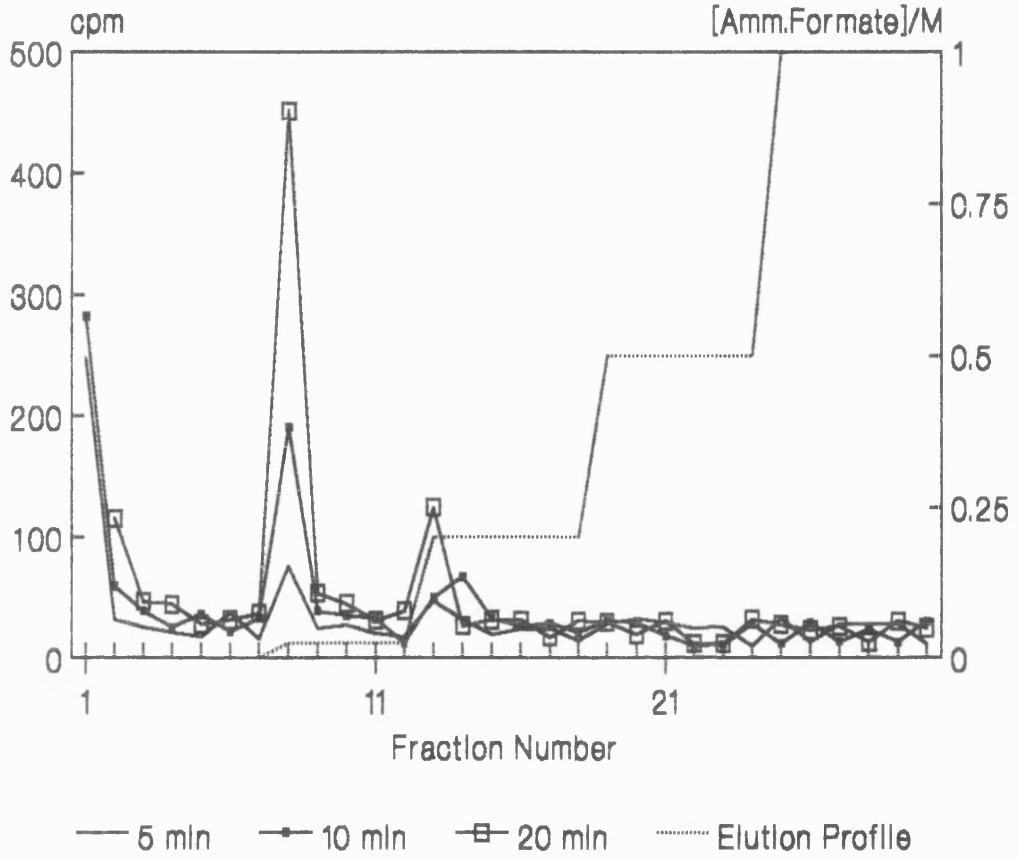


Figure 3.9. The Elution of MC3 Extracts Following "Starvation/Incubation" Treatment.

Cell extracts were collected by centrifugation and applied to a 1 ml Dowex anion exchange column. A stepwise elution system of increasing ammonium formate/0.1 M formic acid concentration was used to elute the compounds and 2 ml fractions were collected manually. Fractions were mixed with 8 ml of Optiphase 'Safe' scintillation fluid and radioactivity was determined by liquid scintillation spectrometry.

3.10). Inositol bisphosphate had not previously been detected in yeast cell extracts (Kaibuchi *et al.*, 1986) and it was noted that the IP_2 peak decreased in magnitude with time whereas both IP_1 and GPI showed an increase. This result inferred that a breakdown pathway was affecting the observed compounds.

Concerned that the starvation treatment may have inhibited cell metabolism or induced a state wherein inositol uptake was impossible, an experiment was performed to compare:-

1] Labelling without starvation, and
2] Normal starvation methodology.

In the normal method (2), incubations of 30 and 60 minutes produced peaks in the GPI, IP_1 and IP_2 windows which increased with time. No IP_3 was seen. Labelling without starvation (1) also demonstrated GPI-IP_2 peak production, but not of the same magnitude. Accumulation with time was not shown but this experiment suggested that a similar result could be achieved without imposing metabolic stress, such as starvation, on the cells. It was therefore possible to consider the use of cells that were wild type for inositol uptake in such experiments.

It was also considered possible that with only radioactive inositol present in the medium, the cells were not provided with an adequate supply of the substrate for full metabolism. Incubations performed in medium enriched with

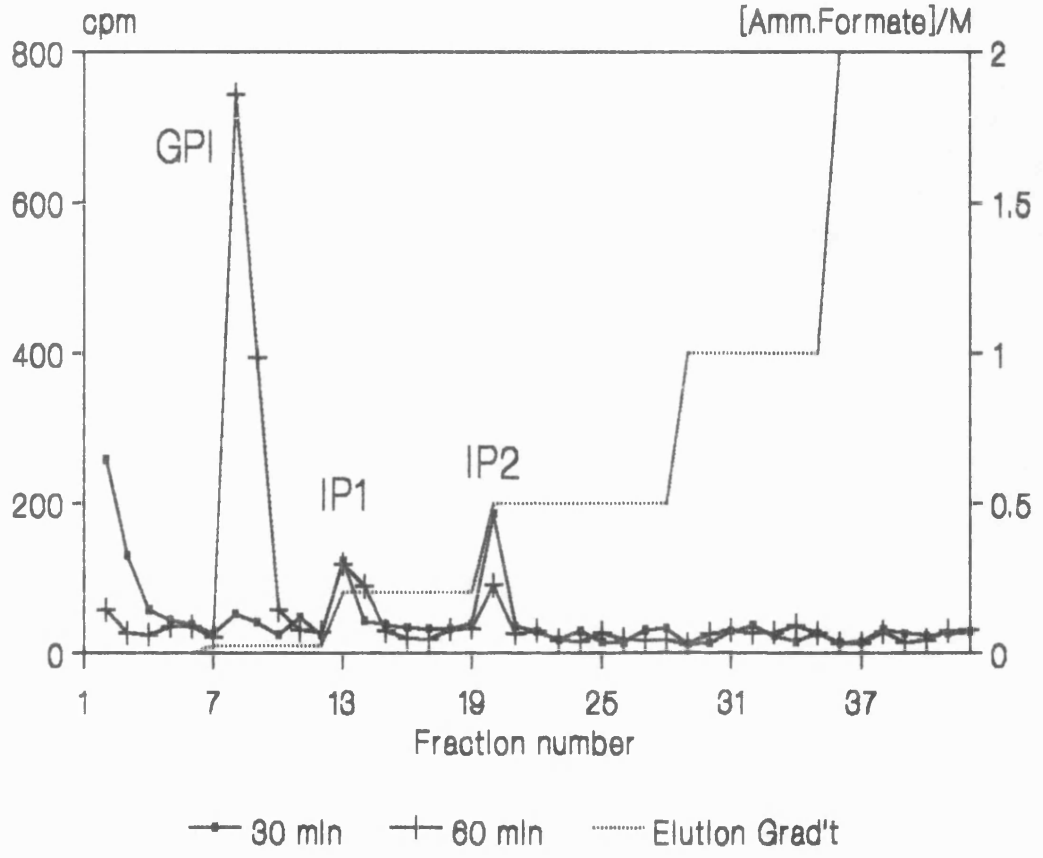


Figure 3.10. The Elution of MC3 Extracts Following Extended 'Starvation/Incubation' Treatment.

Samples of MC3 were treated according to the 'starvation/ incubation' methodology and incubated with myo-[^{3}H]-inositol + PT6-271 for 30 and 60 min. Reactions were halted by boiling (2 min) and extracts were eluted from Dowex anion exchange resin using increasing concentrations of ammonium formate. Fractions (2 ml) were mixed with Optiphase 'Safe' (8 ml) for liquid scintillation counting . Essentially identical results were obtained in duplicate experiments.

added non radioactive inositol did not affect peak formation (data not shown).

The normal method (2) has been repeated with ^{14}C-labelled inositol and the same peaks detected (data not shown).

Earlier experiments had generally involved the incorporation of only very low levels of tracer inositol promoting theories of inadequate labelling concentrations for IP_3 detection. A starvation/incubation assay was performed with 10 $\mu Ci\ ml^{-1}$ ^3H-inositol (no contaminants present) and similar peaks were detected as in previous experiments (Fig. 3.11).

In an attempt to investigate the effect of a glucose/ nutrient stimulation on the system, the starvation period was replaced by resuspension of the cells in a complex medium, i.e. YEPD. Peaks corresponding to GPI and IP_1 were recorded in duplicate experiments. Although these peaks were of a greater magnitude than those obtained in the 'starvation/incubation' experiments, IP_2 was not present. The glucose/nutrient mediated hydrolysis of membrane PIP_2 to produce IP_3 had also not been demonstrated.

To examine the effect of a glucose-only stimulus, the original method was used with an added 1 hour incubation in the presence of a final concentration of 25 mM glucose. Reactions were halted as previously described and the supernatants were analysed by Dowex anion exchange

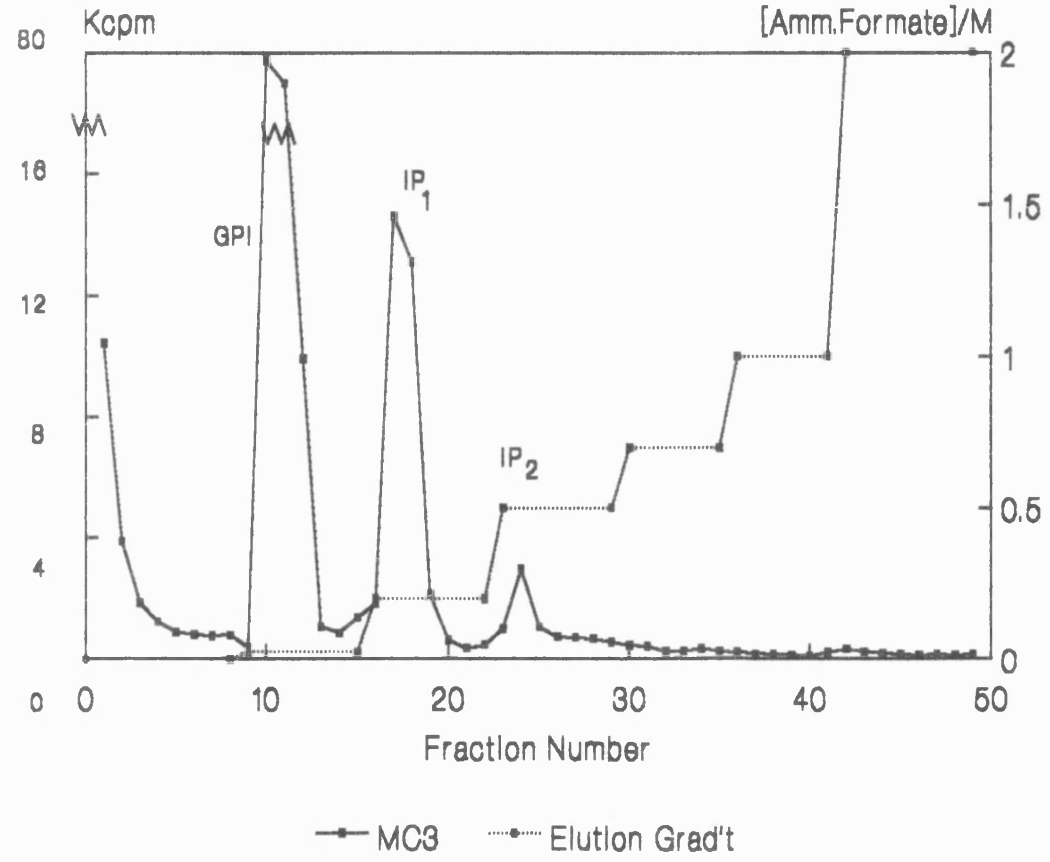


Fig. 3.11. Conversion of ^{3}H-Inositol Experiment using Starvation/Incubation method for 60 min at 10 µCi ml^{-1}

A sample of MC3 was treated according to the 'starvation/ incubation' methodology and incubated with tracer myo-[^{3}H]-inositol + PT6-271 (10 µCi ml^{-1}) for 60 min. Reactions were halted by boiling for 2 minutes and the extract was eluted from a Dowex anion exchange column using a stepwise gradient of increasing ammonium formate concentrations. Fractions were collected and mixed with 8 ml Optiphase 'Safe' fluid for counting by liquid scintillation spectrometry.

chromatography. Three separate experiments gave almost identical elution profiles containing GPI, IP_1 and IP_2 peaks (Fig. 3.12). No IP_3 was detected however and the use of Bligh and Dyer or TCA extraction methods (Berridge *et al.*, 1983) as alternative extraction procedures did not enhance or improve the results in repeat experiments.

Analysis of the initial supernatants (MM ino^- cell free extracts) by elution from Dowex anion exchange columns had regularly shown the presence of both inositol and GPI. This was not unexpected since inositol and GPI account for virtually all the [^{3}H] label in the medium from yeast cultured in the presence of [^{3}H]-inositol (Angus and Lester, 1972). Phosphatidylinositol (PI) catabolism leads to the accumulation of GPI in the medium as it deacylates at the cell surface (Angus and Lester, 1975). Under steady-state conditions, extracellular GPI produced by a growing culture contains about 25% of the inositol and phosphate found in PI itself, although the formation of extracellular GPI from PI turnover is influenced by the availability of an energy source. When cells are deprived of glucose, the formation of GPI declines whereas the appearance of free inositol in the growth medium increases. This situation is reversed by refeeding glucose to the cells (White *et al.*, 1991; Angus and Lester, 1975).

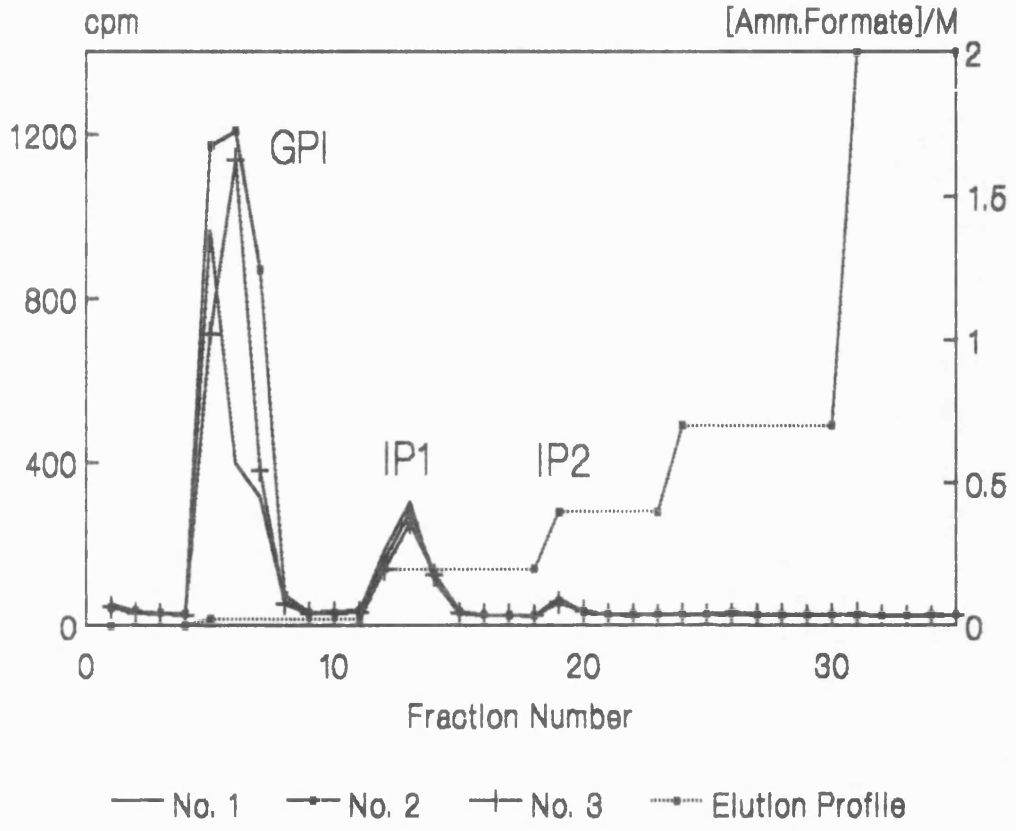


Fig 3.12. Starvation/Incubation Experiment with Glucose Stimulation

Strain MC3 was grown to late exponential phase in YEPD then harvested and washed. The pellet was resuspended in MM ino^- (100 ml) and incubated for 2 hours at 30 ^oC. The cells were then resuspended in 9 ml of MM ino^- and supplemented with ^{14}C-inositol (10 µl, 0.25 µCi). The cells were incubated for 1 hour, then stimulated with glucose (25 mM). Reactions were halted by boiling after a further 60 min and supernatants were analysed by Dowex anion exchange chromatography.

3.6 Incorporation of [^{3}H]-Inositol into *Sacch. cerevisiae*

Prompted by the inability to detect IP_3 in a yeast cell extract, it was decided to monitor the uptake of tracer inositol in a growing culture of the inositol-requiring mutant MC3. It would thus be possible to demonstrate that cells were being labelled to an adequate activity level and provide information about the length of time required for radioactivity incorporation.

The original experiment showed that the total counts in the pellet mirrored the growth curve (Fig. 3.13). This gave good evidence that the yeast cells were actively taking up the tracer as predicted by Henry (1982) and Nikawa *et al.* (1982). The amount of radioactvity in the pellet material was seen to increase with time although the levels in the supernatants remained relatively constant throughout (data not shown). This was thought to be due to the flux between uptake of radioactive inositol from the medium and the extracellular release of ^{3}H-GPI plus PI-derived ^{3}H-inositol.

Observing the exponential phase of growth, which coincided with the period of maximal tracer uptake, the result indicated that a population of exponentially growing cells could be fully labelled within a period of 5-6 hours.

A second uptake experiment was performed using the same methodology, except that growth was monitored by the measurement of cell number using an electronic particle

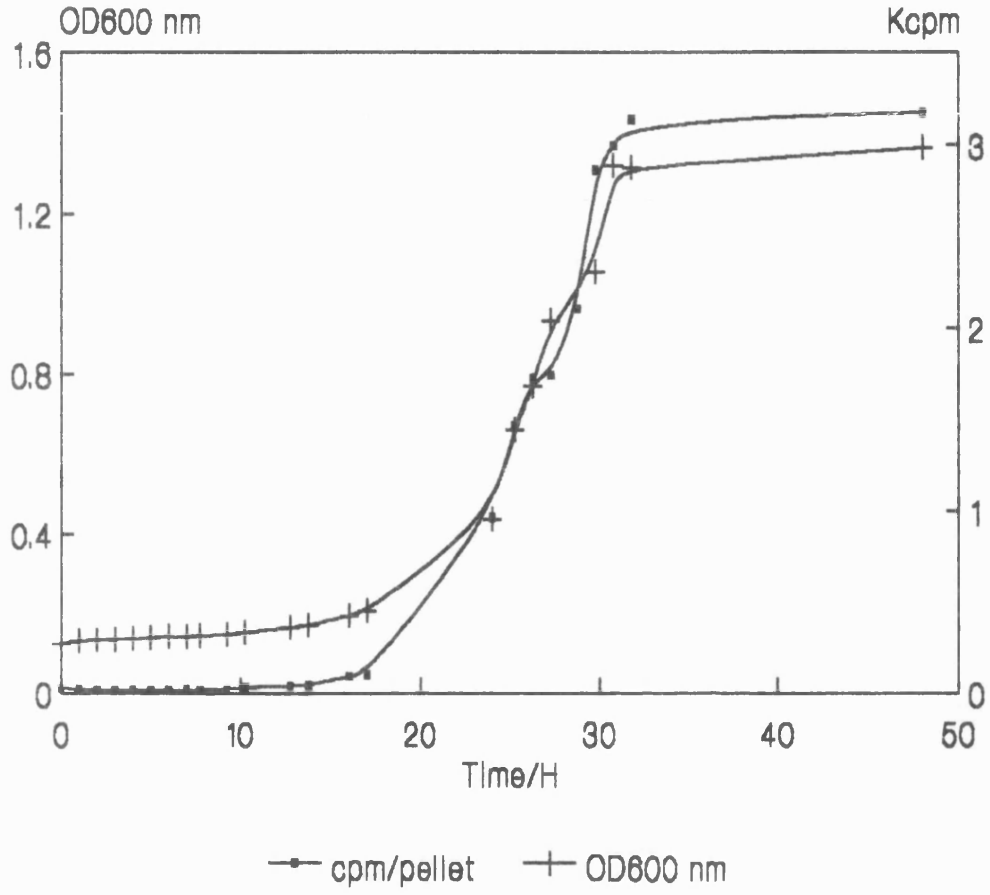


Fig. 3.13. Uptake of ^{3}H-Inositol by *Sacch. cerevisiae* (MC3)
Strain MC3 was grown in MM ino^{+} at 30 ^{o}C with 2 µCi of myo-[^{3}H]-inositol. Samples (2 x 1 ml) were periodically removed and harvested by centrifugation. The pellet was washed once in water and resuspended in 2 ml of water prior to mixing with 8 ml of Optiphase 'Safe' for liquid scintillation counting. Samples (1 ml) were simultaneously removed from a parallel culture and assessed for growth stage by measurement of the optical density at 600 nm.

counter. Once again, total radioactivity in the pellet mirrored the growth curve, but it was also possible to examine the radioactivity incorporated on a 'per cell' basis. Figure 3.14 indicated that inositol uptake may involve the equilibration of internal inositol-sink pools, as a two-stage cell labelling curve is seen. Both experiments demonstrated that maximum labelling had been achieved at the end of exponential phase and the radioactivity per individual cell had maximised by mid-exponential. Any increase in the total amount of radioactivity recovered from a pellet after a cell had reached stationary phase would not be due to further uptake. Once the whole population had reached stationary phase, total uptake would be complete.

Having demonstrated that radioactivity was incorporated, it was then important to show that the ^{3}H-inositol was distributed to the pertinent lipids, PI, PIP and PIP_2. If the phosphoinositides remained unlabelled, it would be impossible to produce radioactive IP_3. A number of possible destinations were available for incorporated myo-inositol that would reduce the efficiency of polyphosphoinositide labelling:-

1) It was reported that myo-inositol gets incorporated into numerous membrane glycoproteins (Conzelmann *et al.*, 1990), the myo-inositol being attached to the glycoproteins as part of a phospholipid moiety which resembles glycophospholipid anchors in other organisms. This would reduce the amount of

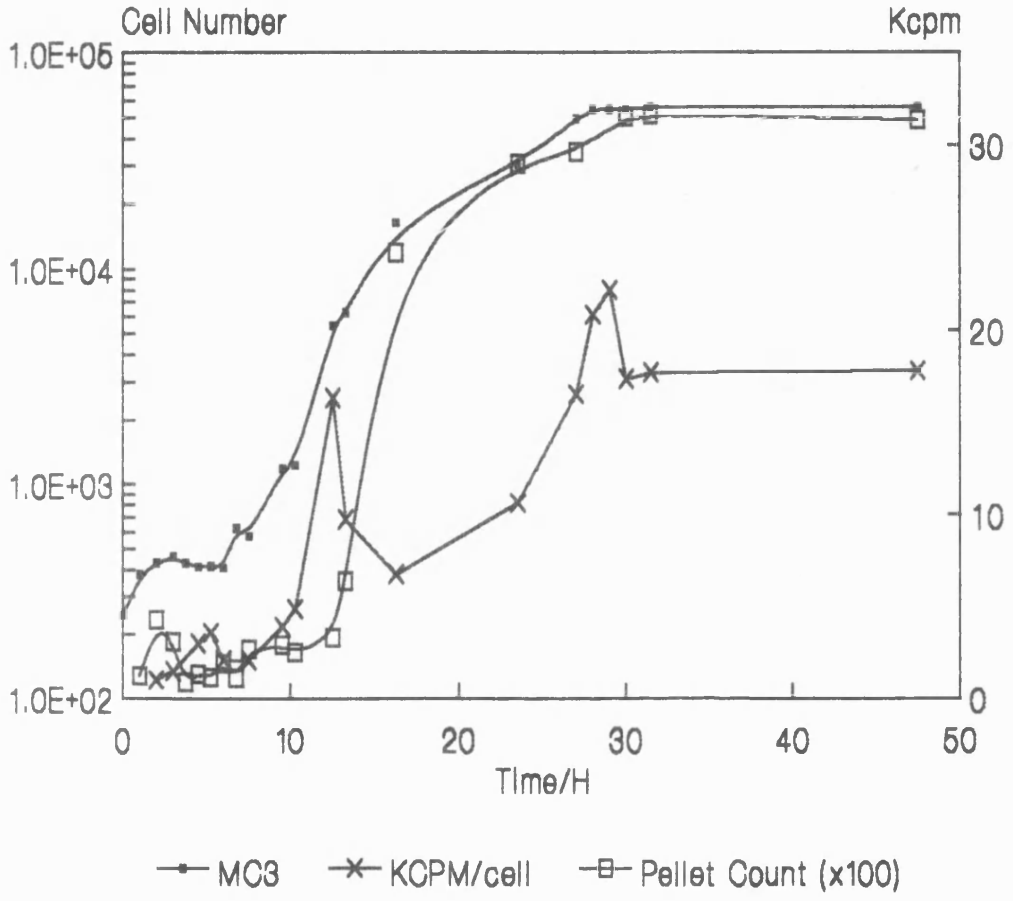


Fig. 3.14. Inositol Uptake Experiment No. 2

Strain MC3 was grown in MM ino$^+$ at 30 ^{o}C with 2 µCi of myo-[^{3}H]-inositol. Samples (2 x 1 ml) were periodically removed and harvested by centrifugation. The pellet was washed once in water and resuspended in 2 ml of water prior to mixing with 8 ml of Optiphase 'Safe' for liquid scintillation counting. One millilitre samples were simultaneously removed from a parallel culture and assessed for growth stage by measurement using the electronic particle counter.

radioactivity available for the polyphosphoinositides as the phospholipid moieties can be added to proteins in the endoplasmic reticulum which are then transported to the Golgi by the regular secretory pathway.

2) Although PI is the major inositol-containing lipid in yeast, inositol-containing sphingolipids represent 40-60% of the total inositol-containing lipids in yeast, depending on growth conditions and strains (Lester *et al.*, 1978). The most abundant inositol-containing sphingolipid in yeast is ceramide-(P-inositol)$_2$ mannose (CerI_2P_2M; Steiner *et al.*, 1969) and studies of the labelling behaviour and turnover of inositol-containing lipids suggests that the inositol in CerI_2P_2M is derived from PI (Angus and Lester, 1972).

3) The previously discussed GPI (Chapter 3.5) would also result in a reduction of available radioactivity for the polyphosphoinositides.

The detection and separation of phospholipids was performed using thin layer chromatography as described in Materials and Methods 2.22 after Medh and Weigel, (1989); Auger *et al.*, (1989a); Billah and Lapetina, (1982) and Jolles *et al.*, (1981). Extracted lipids were initially filtered through cellulose nitrate filters to remove yeast solids, but this was believed to inhibit the recovery of lipids as they became stuck in the filter matrix (Veazey, F., 1990; pers. comm.). Filtration was therefore performed with glass microfibre filters (GF/F, Whatman, UK). Test plates were

spotted with 5-6 µl of known unlabelled phospholipid standards and the mobile phase of Billah and Lapetina (1982) was used to separate them. Three attempts were performed in chromatography tanks that had not been lined with filter paper. No separation of the standards was detected although 'tracks' were visible on the plate. The phospholipids had been moved by the mobile phase but no separation had been achieved. Believing the fault to be due to non-compatibility of the mobile phase, the method of Jolles *et al.* (1981) was used. Similar results were obtained in duplicate experiments. Tracks were visible on the plate and no distinct spots could be identified. It was concluded that due to non-saturation of the chromatography-tank atmosphere, the solvent was continuously evaporating from the surface of the plate. At a certain point, the rate of evaporation was equal to rate of mobile phase travelling up the plate. This made further progress up the plate extremely slow or impossible. The lipids however continued to move upwards and converged at the level of the solvent front. It was for this reason that in many cases only one large stained area appeared at the height of the solvent front. Chromatography tanks were therefore lined with filter paper before addition of the mobile phase and allowed to equilibrate for at least 3 hours prior to introduction of a plate for development.

This modification allowed separation of the standard phospholipids. The Rf values altered slightly according to the period of development in the tank and the ambient

temperature but the order of elution remained constant: Phosphatidic acid (PA) > Phosphatidylserine (PS) > Phosphatidylcholine (PC) > Phosphatidylinositol.
The lipid extract from a non-labelled culture of strain MC3 grown to late exponential phase was used in a trial run in the same chromatographic conditions. Observing four separate bands in the extract, one of which eluted with the PI standard, the separation was repeated with a culture (100ml) grown to late-exponential phase in the presence of 2 µCi of myo-[^{3}H]-inositol + PT6-271. Four bands were again recorded - two eluted with standards PI and PS and two did not correspond to any of the standards.

The bands and inter-band regions were cut out and mixed with scintillation fluid (8 ml) for detection of radioactivity. Of the radioactivity recovered from the plate 73.4% was located in the PI band (Rf 0.5). This was positive evidence for successful ^{3}H-inositol uptake and incorporation into the phosphoinositides. Two further areas of radioactivity were also detected on the plate, a visible band at Rf 0.29 contained 8.12% of the recovered radioactivity and an inter-band area at Rf 0.45 was labelled with 10.1% of the total radioactivity. Neither of these areas eluted with the standards, and none of the other bands contained any radioactivity. The 'secondary' bands were thought to represent radioactivity incorporation into the polyphosphoinositides.

Unlabelled PIP_2 was obtained and the lipid extract from a non-labelled culture was eluted using PI, inositol and the polyphosphoinositide as standard markers. The expected four bands were obtained from the extract, none of which co-eluted with PIP_2. The PIP_2 marker did not elute in the position of either of the two previously detected radioactive bands and it was considered possible that those areas of radioactivity were derived from lipid-soluble moieties of the radiolytic decomposition products of ^{3}H-inositol. No evidence could be provided for this however, so to eradicate the possibility of contamination a repeat labelling experiment was performed with ^{14}C-inositol.

Unlabelled PIP was also obtained and a test plate was run in conjunction with an unlabelled yeast lipid extract to assess the separation of the phosphoinositide markers. The phosphoinositide standards eluted in the order PI > PIP > PIP_2 with the Rf values 0.55: 0.37: 0.16. The characteristic four bands were present in the lipid sample and it was interesting to note that the band previously found to contain radioactivity co-eluted exactly with PIP. It was concluded that radioactivity had been successfully incorporated into both PI and PIP in the ^{3}H-inositol labelling experiment, and the counts in the unidentified bands were not due to decomposition products.

A duplicate experiment was performed in which 100ml cultures of strain MC3 were labelled with 2 µl (0.05 µCi) of ^{14}C-inositol and grown to late exponential/stationary phase.

Lipids were extracted and analysed in the usual manner but the results were inconclusive. Although a definite area of radioactivity, which co-eluted with PI could be isolated from both plates, it was impossible to define any other bands because the labelling concentration was not high enough. Apart from the PI bands, the two plates showed no common features and it was difficult to distinguish between labelled areas and background counts. To remedy this situation, a 400 ml culture of strain MC3 was grown to late exponential phase with 0.25 µCi of ^{14}C-inositol and harvested for lipid extraction. It was then possible to detect three radioactive bands which co-eluted with the phosphoinositide standards. Of the counts recovered from the plate 63.7% were located in the PI band, 10.15% were in the PIP band and 2.58% were in the area corresponding to the PIP_2 band.

Although the relevant membrane phospholipids had been isolated and separated, they were not found in the 1:4:140 molar ratio (PIP_2:PIP:PI) described by Steiner and Lester (1972b). The observed ratio was 1:6:25 which compared more favourably with the 1:7:78 ratio described by Kaibuchi *et al.* (1986). It is not known whether the differing ratios were an aspect of different strains, different growth conditions or variations caused by the extraction technique.

In a similar experiment performed at a higher labelling concentration (7.5 µCi ml^{-1}, myo-[^{3}H]-inositol), the same bands were recorded but in a PIP_2:PIP:PI ratio of 1:5.6:59.2

(PI band contained 127,200 cpm). The variation in the observed molar ratios could possibly be attributed to experimental error but Berridge (1984) states that the kinases and phosphomonoesterases that maintain the dynamic equilibrium between these three lipids are some of the most active in the (mammalian) cell. A change in any one of the trio will be rapidly 'buffered' by interconversion of the other two participants (Hawthorne and Pickard, 1979). It would seem that changes in the levels of phosphoinositides occur constantly in the cell according to environmental conditions, growth stage and stimuli which may explain the different results

Wishing to examine the labelling of the inositol phospholipids in a more precise manner, the lipid extracts of labelled yeast cultures were deacylated according to the method described in Materials and Methods 2.16. The extracts could then be analysed by HPLC as detailed in Materials and Methods 2.17. Believing the HPLC apparatus to be capable of detecting lower concentrations of phosphoinositides than TLC, initial labelling were performed at relatively low tracer concentrations (^{14}C-inositol, 0.25 µCi). Elution of samples from both mid-exponential and stationary phase cells showed only a single peak corresponding to PI. Polyphosphoinositide peaks were not present (Fig. 3.15). A labelling experiment was therefore performed with myo-[^{3}H]-inositol at a concentration of 20 µCi ml^{-1}. The cells were grown to late exponential/stationary phase before they were harvested for lipid extraction. The elution profile showed

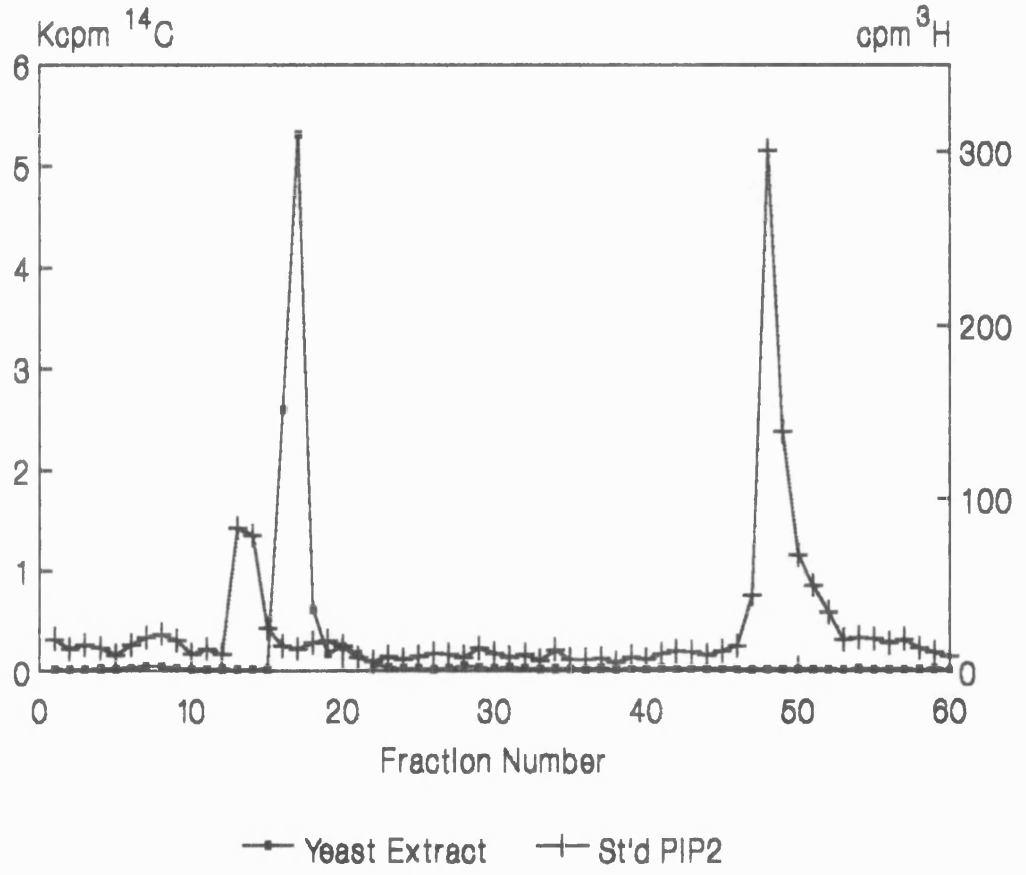


Fig. 3.15. Elution of Deacylated ^{14}C-Labelled Lipid Extract

A late exponential/stationary phase culture of strain MC3, grown in the presence of 0.025 µCi of ^{14}C-inositol was harvested by centrifugation and subjected to a lipid extraction. The extracted lipids were deacylated as described in Methods and Materials 2.16 and analysed on a 10µm SAX Partisil HPLC column using the elution regime described in Methods and Materials 2.17. Ten microlitres (0.1 µCi) of standard ^{3}H-PIP_2 were treated in the same way and eluted as a marker.

three peaks, two of which corresponded to PI and PIP (Fig. 3.16) but no PIP2 was present. The third peak eluted in the position of PS but was not believed to represent this lipid and remained unidentified. Further data were not considered essential for the progression of this part of the project as the labelling, detection and isolation of the inositol-phospholipids had already been shown using TLC.

3.7 Investigation of the Work of Kaibuchi *et al.* (1986)

There was only one published report of the detection of IP_3 from *Sacch. cerevisiae*. Kaibuchi *et al.* (1986) stated that incubation of yeast at very low (0.02% w/v) glucose levels led to the arrest of the cell cycle at the G_0/G_1 phase. Readdition of glucose to these 'starved' cells resulted in cell proliferation. Furthermore, preincubation of yeast with [^{3}H]-inositol and subsequent exposure to glucose resulted in rapid formation of [^{3}H]-inositol monophosphate and [^{3}H]-inositol trisphosphate, presumably derived from phosphatidylinositol and phosphatidylinositol bisphosphate.

Since appropriate protocols had not demonstrated the presence of IP_3 in a yeast cell extract, it was decided to repeat as exactly as possible the experiments of Kaibuchi *et al.* (1986) to confirm their observations and provide further evidence for the existence of the second messenger. The method is described in Materials and Methods 2.12 and involved the collection of 2 supernatants; the primary supernatant was collected after incubation of cells with

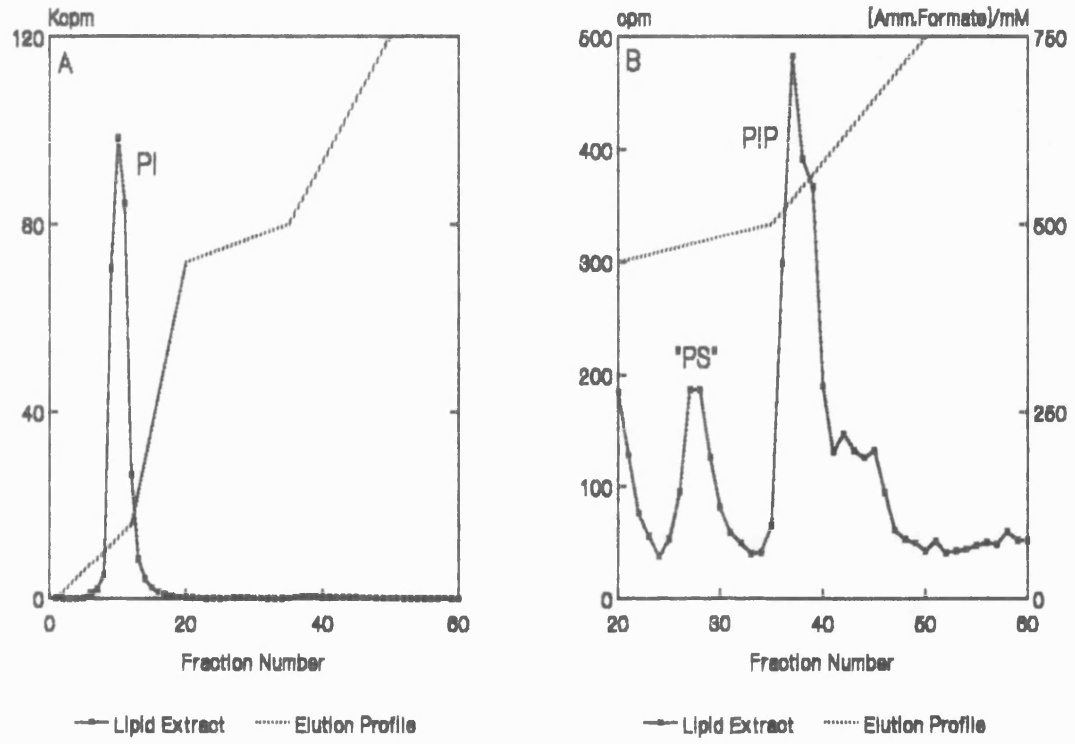


Fig. 3.16. Elution of Deacylated ^{3}H-Labelled Lipid Extract

Strain MC3 was grown to late exponential phase in the presence of ^{3}H-inositol (20 µCi ml^{-1}). The cells were harvested by centrifugation and washed in water (10-20 ml), lipids were extracted and deacylated using the method described in Methods and Materials 2.16. The extract was eluted from a 10 µm SAX Partisil HPLC column using the method described in Methods and Materials 2.17. A, shows the whole profile. B, shows the second half of the profile which is effectively masked by the huge PI peak.

tracer inositol but before stimulation. The secondary supernatant was collected after glucose-stimulation of the labelled cells and it was in this fraction that the detection of radioactive inositol phosphates has been reported.

Strain MC3 was used throughout the repeats of these experiments as it had a similar genotype to the strain MC13 (MATa *ade1 ino1-13*) used by Kaibuchi *et al.* (1986). In the initial experiment, cells were resuspended in 4 ml of MM ino^+ medium together with 40 µl of myo-[2-^{3}H]-inositol + PT6-271 (10 µCi ml^{-1}) and incubated at 30 ^oC for 24 hours with shaking (120 RPM). The pellet was resuspended in 4 ml of the stimulating buffer (Tris/HCl pH 7.0, 25 mM glucose), and was incubated for 2 hours at 30 ^oC. No trisphosphate was detected in either supernatant, however a large peak was present in the "lower phosphate" window of both profiles (Fig. 3.17). A repeat experiment was performed with the intention of making a fuller investigation of the radioactivity content of the supernatants. The aim was to determine whether GPI or IP_2 was contributing the most to the lower phosphate peak. IP_2 can be produced from the breakdown of IP_3 by phosphatase activity or from PIP by PLC activity, so an abundance of the bisphosphate might have indicated rapid IP_3 production and subsequent degradation.

GPI is produced directly from the deacylation of PI by phospholipase A and its presence in high concentrations (in the absence of inositol phosphates) would have implied that

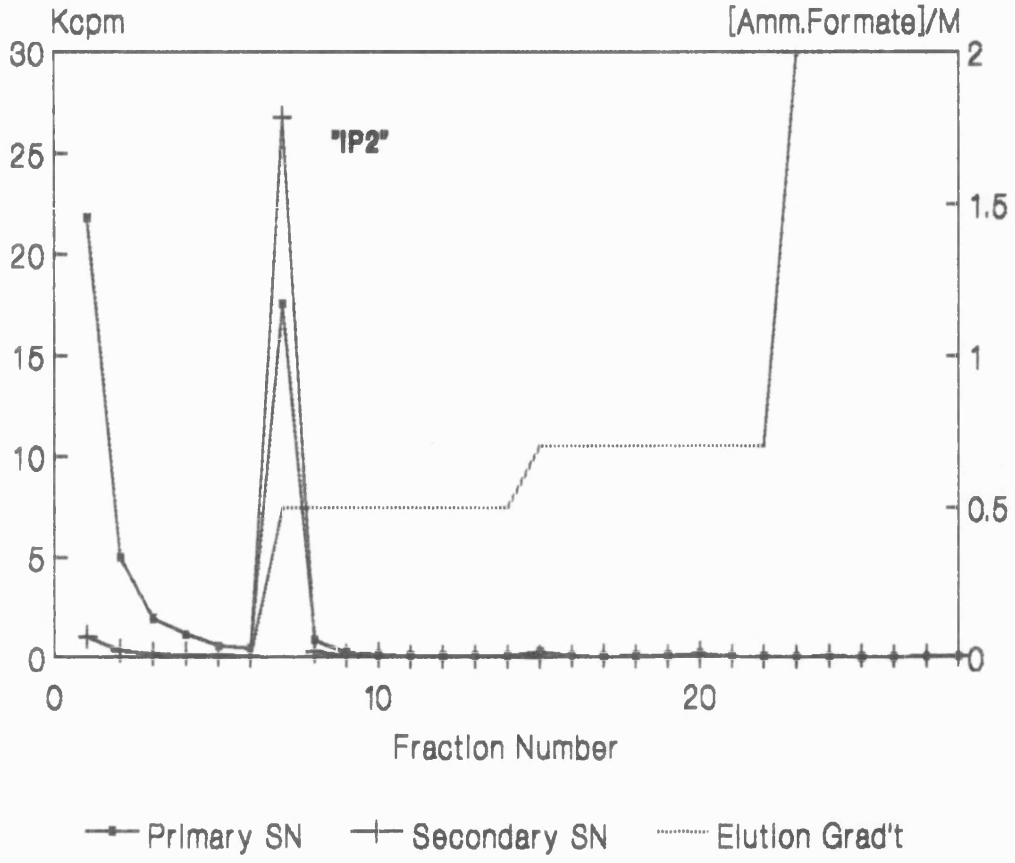


Fig. 3.17. Elution of Supernatants from Glucose Stimulated PIP_2 Hydrolysis Experiment

The primary and secondary supernatants from a repeat of the experiment of Kaibuchi *et al.* (1986) were collected as described in the text and eluted from Dowex anion exchange columns. [^{3}H]-inositol was removed by washing the column with water and the lower inositol phosphates (GPI, IP_1 and IP_2) were eluted together to clear the column. Higher inositol phosphates were eluted with increasing concentrations of ammonium formate/formic acid.

turnover as seen in mammalian cells was not occurring in yeast. The repeat involved a slight change in the methodology, to represent more accurately the method of Kaibuchi *et al.* (1986) - having removed the primary supernatant, the pellet was resuspended and incubated in Tris/HCl pH 7.0 without glucose. After 2 hours, a glucose stimulus was introduced (final concentration 25 mM) and reactions were halted by boiling 45 minutes later. The secondary supernatant was collected as before, and both supernatants were eluted from Dowex anion exchange columns.

The analysis of the primary supernatant gave an unexpected result (Fig. 3.18). The profile showed peaks corresponding to free inositol, GPI, IP_1 and IP_2. It had been assumed that [^{3}H]-inositol and [^{3}H]-GPI would be present in this fraction (see Results 3.5), but the appearance of IP_1 and IP_2 could not be accounted for. Even if active turnover had been occurring during the 24 hour incubation period, the presence of water-soluble intermediates outside the cells in the medium was unexpected. It was proposed that in a repeat experiment, the cell harvest at this stage could be performed by filtration to avoid possible damage to the yeasts.

The elution profile of the secondary supernatant was dominated by a pronounced and unexpected peak in the IP_1 position. There was no evidence of an IP_3 peak, but very small GPI and IP_2 peaks were present. The IP_2 peak was effectively masked by the large IP_1 peak (Fig. 3.19), but it

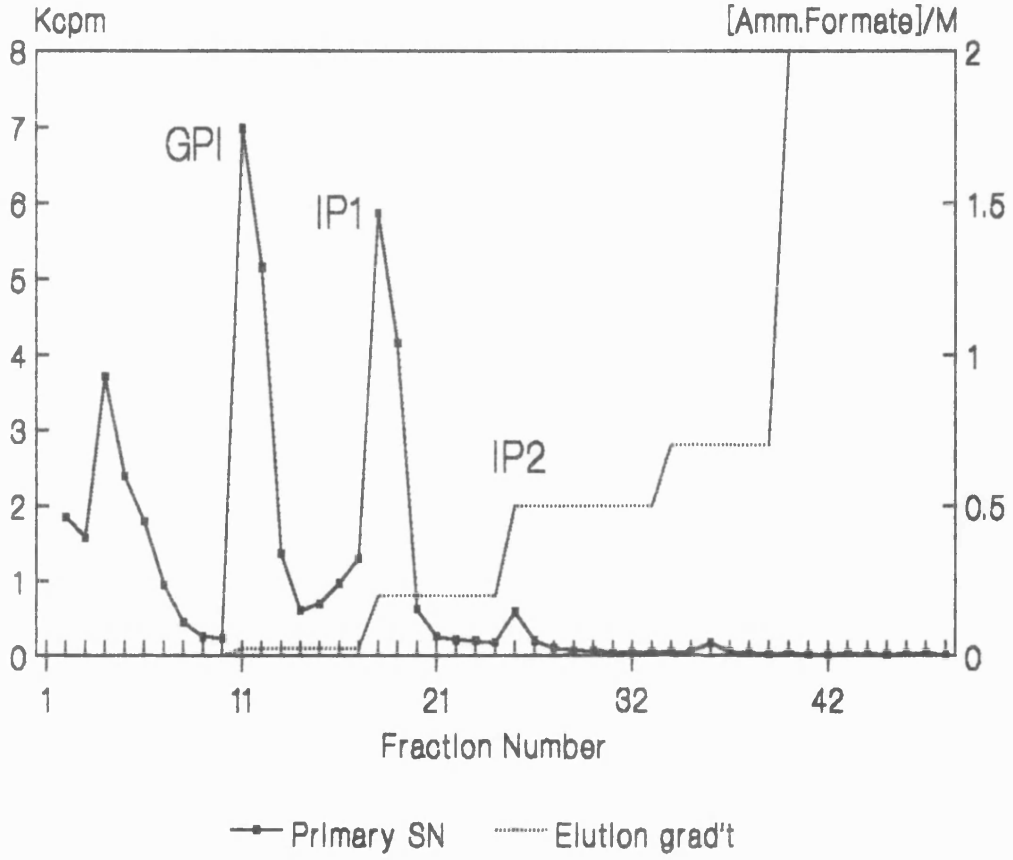


Fig. 3.18. Elution of Primary Supernatant from Glucose Stimulated PIP_2 Hydrolysis Experiment

Cells were grown to mid-exponential phase and harvested by centrifugation. The pellet was resuspended in MM ino^+ (4 ml) with myo-[2-^{3}H]-inositol (10 µCi ml^{-1}) and incubated for 24 h at 30 ^oC. The cells was harvested by centrifugation and the supernatant eluted from a Dowex anion exchange column with increasing concentrations of ammonium formate/formate acid. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' for liquid scintillation counting.

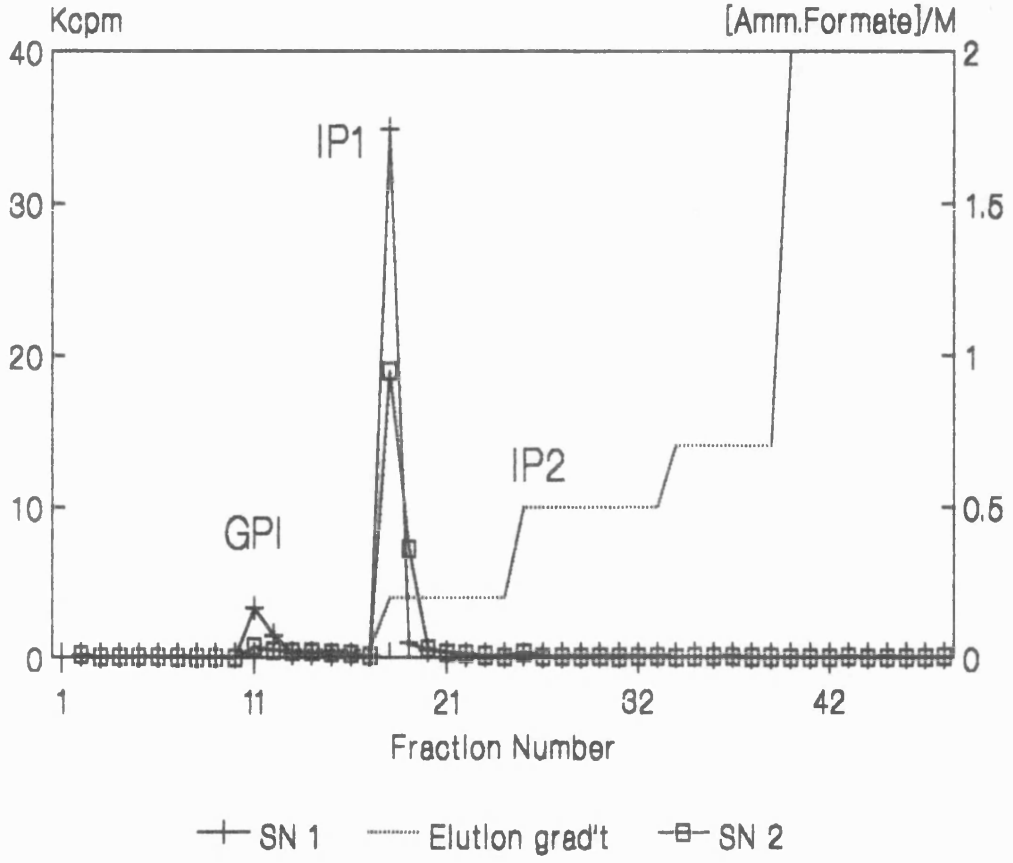


Fig. 3.19. Elution of Secondary Supernatants from Glucose Stimulated PIP_2 Hydrolysis Experiments

Secondary supernatants were obtained from repeats of the experiments first performed by Kaibuchi *et al.* (1986) after halting reactions by boiling the samples for 2 min. The supernatants were removed after centrifugation of the mixtures and eluted from Dowex anion exchange columns using increasing concentrations of ammonium formate/formic acid. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by scintillation counting.

could be seen if the IP_1 peak was removed (data not shown). The detection of the monophosphate peak corresponded with the observations of Kaibuchi *et al.* (1986) but the concomitant trisphosphate peak was not present. It was considered possible that any IP_3 produced had already been degraded by phosphatase activity. According to the source paper however, this does not obtain. Tritiated IP_3 was shown to have have reached its maximum concentration after 30 min,but no sign of degradation was reported even after 60 minutes post-stimulation. No IP_2 detection was reported in the original reference which also argued against the activity of a phosphatase pathway (Kaibuchi *et al.*, 1986). A repeat of this experiment confirmed the production of the observed peaks, and sonication was performed on the pellet prior to removal of the secondary supernatant in an attempt to enhance radioactivity recovery. Apart from the inclusion of harvest by filtration after the 24 hour incubation, and sonication (Amplitude 10 μM, 10 min) of the suspension post-boiling, the methodology remained the same. Compared to the previous experiment, similar peaks were detected in the primary supernatant, and an almost identical secondary supernatant elution profile was recorded (Fig. 3.19) although the total radioactivity counts recovered was slightly less. It was decided to re-examine the tracer material in order to explain the presence of lower inositol peaks in the primary supernatant. Peaks were recorded in the positions of GPI, IP_1 and IP_2. It was not known if the PT6-271 polymer had only a limited period of activity or if the impurity build-up had exceeded its absorption limits, but

contaminant peaks were present in the tracer (Fig. 3.20). By subtracting the appropriately corrected 'background' counts, it was possible to assign the primary supernatant IP_1 and IP_2 counts to the decomposition products. The GPI peak however, was much greater than the contaminant level and could therefore be attributed to genuine metabolism by the yeast cells. In summary, IP_1 and IP_2 had not been recovered from the primary supernatant but the peaks were derived from radiolytic decomposition products that eluted with the same retention properties.

The profiles obtained from the secondary supernatants could not be explained completely by the presence of contaminants. The monophosphate peaks were much too large to be comprised solely of 'background' radioactivity and indicated that some kind of turnover had occurred. No evidence of a trisphosphate peak had been seen and as such, the observations of Kaibuchi *et al.* (1986) could not be repeated.

To eliminate the problem of contaminating species, ^{14}C-labelled inositol was used as the radioactive tracer. A culture was grown to a cell density of 1.82×10^7 cells ml^{-1} then harvested by centrifugation. The washed pellet was resuspended in 100 ml of MM ino^- with 10 µl (0.25 µCi) of ^{14}C-labelled inositol and incubated at 25 ^oC for 24 hours. The use of MM ino^- medium at this stage was an attempt to induce maximal tracer uptake by offering ^{14}C-inositol as the only inositol source. The suspension was reharvested, the

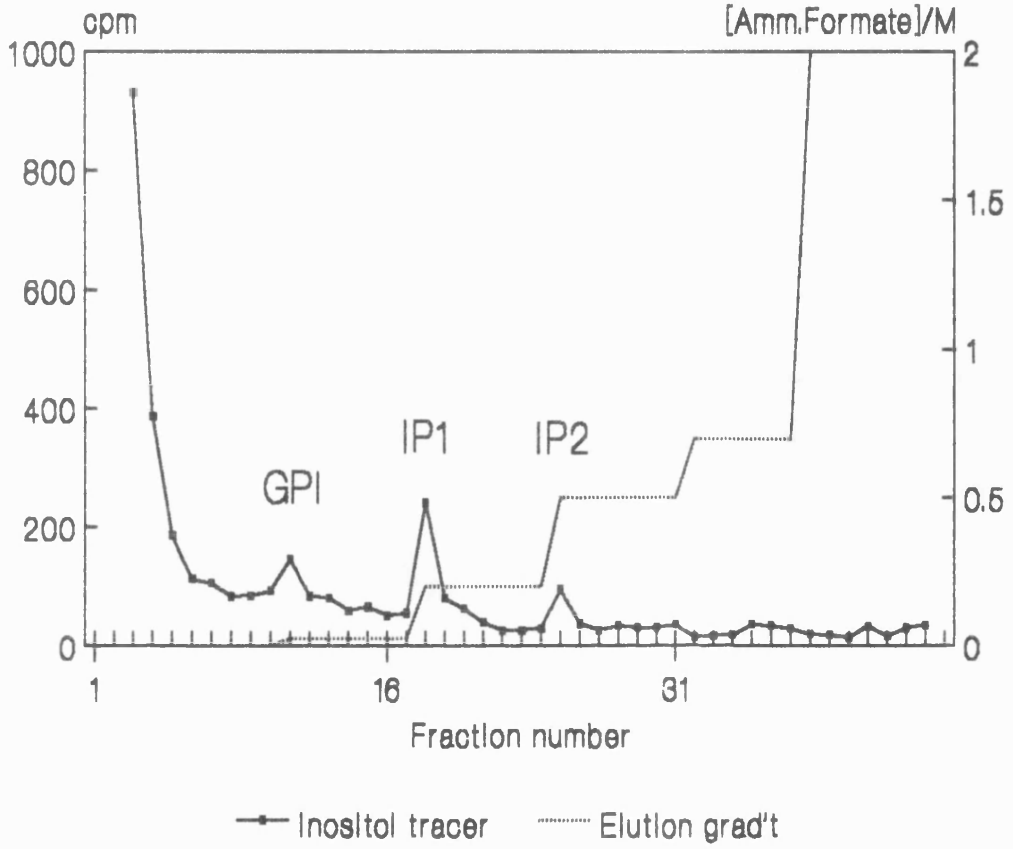


Fig. 3.20. Elution of Myo-[2-^{3}H]-Inositol from a Dowex Anion Exchange Column

One microlitre (1 µCi) of the inositol tracer (+ PT6-271) was diluted in distilled water (2 ml) and eluted from a Dowex anion exchange chromatography column (1 ml) using increasing concentrations of ammonium formate/formic acid. Fractions (2 ml) were collected manually and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation counting. Peaks detected in the profile appeared in the positions of GPI, IP_1 and IP_2.

primary supernatant removed and the pellet resuspended in the 'starvation' buffer (9 ml). The Tris/HCl buffer had been slightly modified to duplicate that of Kaibuchi *et al.* (1986). Described as 'MES buffer', it comprised MES and Tris at both 0.1 M which was then adjusted to pH 6.5. The mixture was incubated for 2 hours before 1 ml of glucose solution was added to a final concentration of 25 mM. Reactions were halted 45 min later by incubating the sample at 100 ^{o}C for 2 min, the yeast cells were pelleted by centrifugation and radioactivity was extracted from the pellet using the Bligh and Dyer (1959) technique. This was considered to be a more exhaustive extraction procedure than that previously used and ensured that any radioactivity recovered was derived only from the pellet material. Elution of the aqueous phase from Dowex anion exchange columns showed only a background radioactivity trace. It was not known if the level of radioactivity in the medium had been too low for adequate incorporation or the growth conditions had been limiting, but no evidence of metabolism was recorded. Analysis of the primary and secondary supernatants indicated that radioactivity had been taken up and metabolized. The primary supernatant showed inositol and GPI peaks, and no other compounds. The secondary supernatant, which had been removed prior to the extraction procedure produced a profile containing three peaks. These unexpectedly eluted in the positions of GPI, IP_1 and IP_2 (Fig. 3.21).

The *prima facie* evidence suggested that actual uptake and turnover of the ^{14}C-inositol had taken place. A repeat of

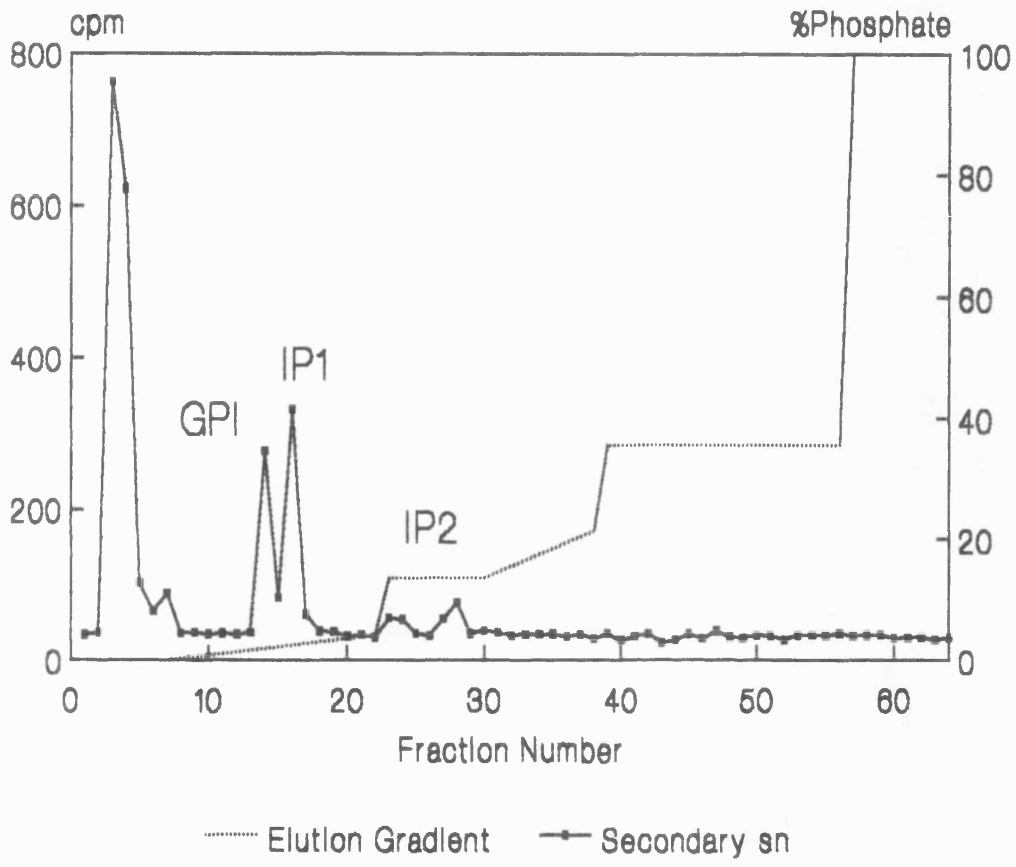


Fig. 3.21. Analysis of Secondary Supernatant from Glucose Stimulated Inositol Turnover Experiment

The secondary supernatant from a glucose-stimulated hydrolysis of PIP_2 experiment was collected following centrifugation, and eluted from a 10 µm SAX Partisil HPLC column using the method of Batty *et al.* (1985, 1989). Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for liquid scintillation counting. Single peaks were recorded in the positions of GPI and IP_1. The IP_2 elution window shows 2 small peaks - indicating 2 isomers.

this experiment was performed using a higher labelling concentration (0.625 µCi in 4 ml of MM ino^+). MM ino^+ medium was used to ensure that the medium was not limiting. Harvesting and treatment of the cells was performed as described in the previous experiment. Elution of the aqueous phase from Dowex anion exchange resin showed the presence of peaks corresponding to GPI, IP_1 and IP_2 (Fig. 3.22). The identities of the peaks were confirmed by repetition and provided evidence for genuine inositol turnover in yeast cells. The profile did not show a trisphosphate peak however so no evidence for the existence of the second messenger could be provided. Analysis of the primary and secondary supernatants showed 'expected' profiles of inositol and GPI, and inositol, GPI, IP_1 and IP_2 respectively (data not shown).

A fresh batch of myo-[2-^{3}H]-inositol + PT6-271 was obtained and a series of labelling experiments were performed at a radioactivity concentration of 20 µCi ml^{-1}. Previous glucose-stimulated turnover experiments had only been performed at a maximum radioactivity concentration of 10 µCi ml^{-1} and it was considered posssible that inositol trisphosphate was not detectable at this level. A concentration of 20 µCi ml^{-1} was selected to mimic exactly the conditions of Kaibuchi *et al.* (1986).

Cells (2 ml from 100 ml) were suspended in 2 ml of a minimal medium (yeast nitrogen base, 0.67% w/v; D-glucose, 4% w/v; myo-inositol, 50 µM; myo-[2-^{3}H]-inositol, 40 µCi) and

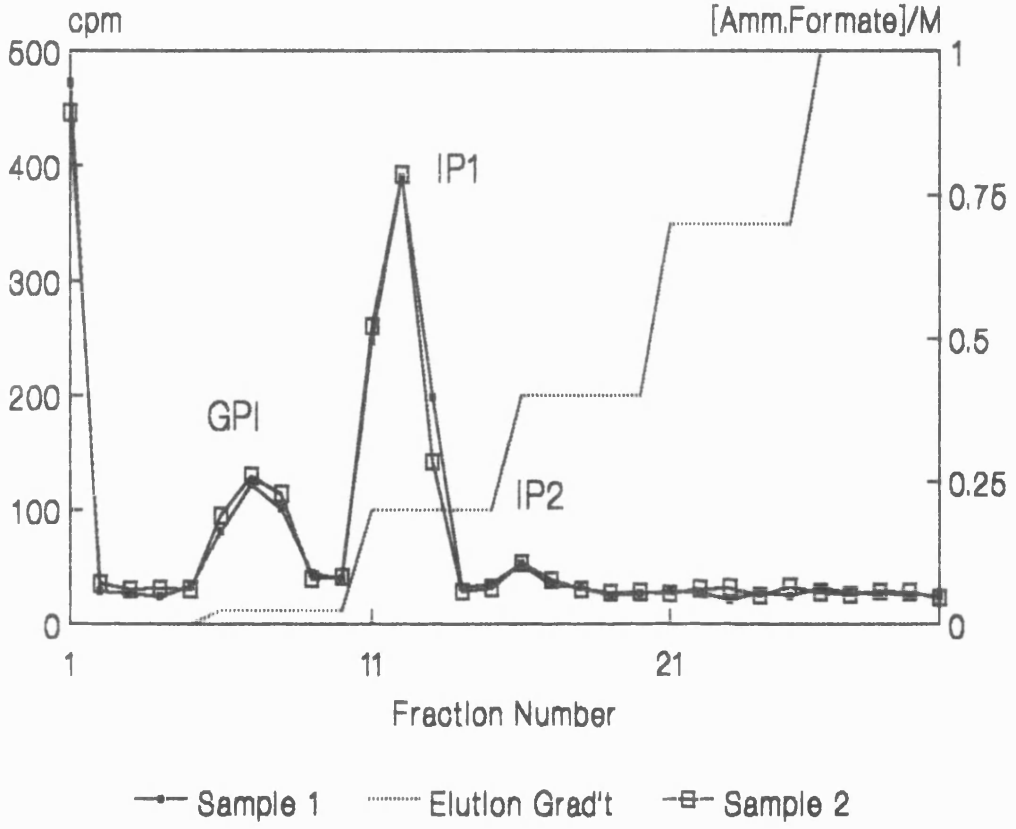


Fig. 3.22. Elution of Aqueous Phases from Extracts of Glucose Stimulated Inositol Turnover Experiments

Following removal of the secondary supernatant, cells were washed in distilled water and radioactivity was extracted by the method of Bligh and Dyer (1959). Aqueous extracts were eluted from Dowex anion exchange resin using increasing concentrations of ammonium formate. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for liquid scintillation counting. In repeat experiments, peaks corresponding to GPI, IP_1 and IP_2 were detected.

incubated for 24 hours at 37 ^{o}C. An elevated temperature was used in an attempt to enhance potentially occurring turnover of the transduction system. Subsequently, it has been shown that non-temperature-sensitive strains show a higher level of response to stimulation at this temperature (Hawkins, P., 1992; pers. comm.). A parallel culture showed an increase in optical density at 600 nm from 0.105 to 0.530 (1 in 10 dilution), indicating that the cells had been proliferating. The cells were harvested and washed as described above, the supernatants were pooled (primary supernatant) and the pellet was resuspended in MES buffer (2 ml). This was incubated at 30 ^{o}C for 2 h when the suspension was reharvested, washed in distilled water and resuspended in fresh buffer (4 ml). The supernatants were pooled as the secondary supernatant. The cell suspension was split into 2 x 2 ml portions, one of which was stimulated by the addition of glucose at a final concentration of 25 mM. Samples (1 ml) were immediately taken from both the stimulated and unstimulated cells, and reactions were halted by the addition of ice-cold methanol:chloroform (2:1 v/v). The remaining cell suspensions were incubated at 30 ^{o}C for a further 45 min, then the reactions were halted as above. Following incubation on ice for 10 min, cells were harvested by centrifugation in microcentrifuge tubes and tertiary supernatants removed. Radioactive metabolites were extracted by the method of Bligh and Dyer (1959); extracts were pooled with the tertiary supernatant and the upper-aqueous layers were analysed by column chromatography.

The result (Fig. 3.23) showed that in both extracts from the stimulated cells, peaks in the positions of inositol and GPI were detected. The inositol peak showed a marked decrease in magnitude during the incubation period whereas the GPI peak showed a coincident increase. This result suggested that turnover had occurred within the cell, but that the phospholipid involved was PI. No evidence of peaks corresponding to the inositol phosphates was present. In the extracts from the non-stimulated cells, only an inositol peak was present at time zero, but peaks in the positions of inositol and GPI were detectable after 45 min. A slight decrease in the magnitude of the inositol peak could be measured but the most prominent feature of the profile was the production of a GPI peak to the same extent as in the stimulated cells. This suggested that exactly the same turnover of inositol phospholipids had occurred in the yeast cells irrespective of the presence of glucose. No lower phosphate peaks were present in any of the extract profiles, which refuted theories of low radioactivity concentration being the cause of non-detection. Lower inositol phosphate peaks were recorded in the experiments performed with ^{14}C-labelled inositol at much lower concentrations. Analysis of both the primary and secondary supernatants by HPLC column chromatography showed the presence of both [^{3}H]-inositol and [^{3}H]-GPI peaks (data not shown). This was an expected result (Results 3.5) and gave evidence for active metabolism in the cells, and for the absence of radiolytic breakdown products. The similarity of the profiles recorded in the primary supernatant, secondary supernatant and cell extract led to a

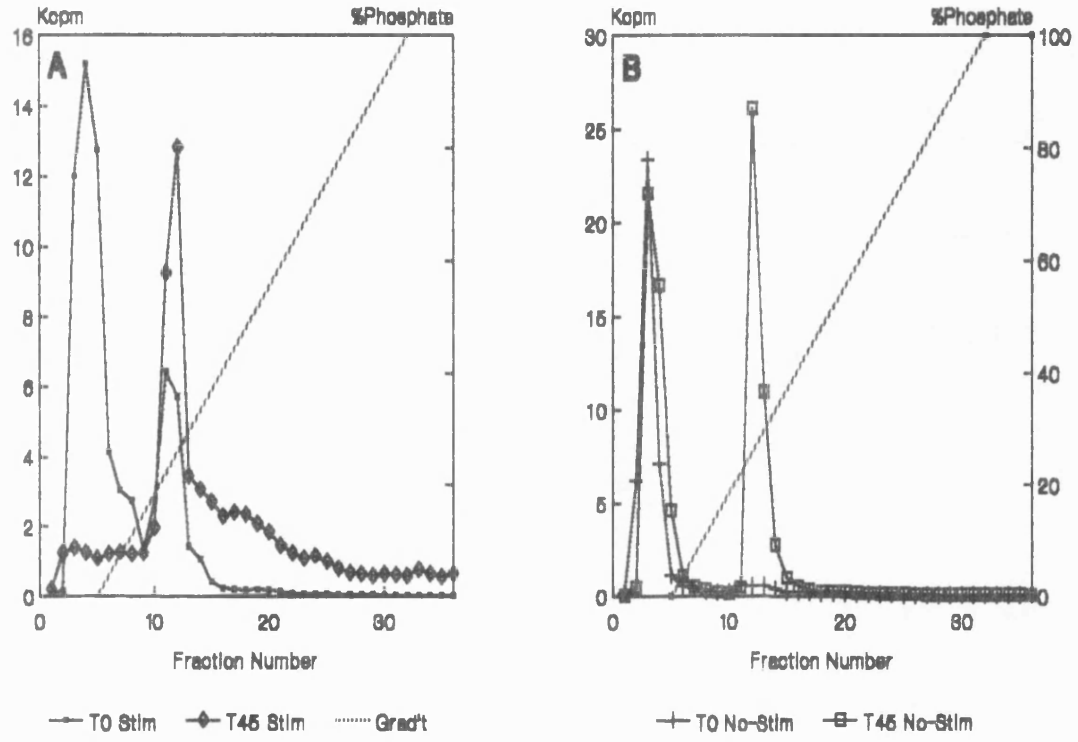


Fig. 3.23. Analysis of Extracts from Stimulated and Non-Stimulated Cells after Glucose Stimulation

Cells grown in the presence of [^{3}H]-inositol (20 µCi ml^{-1}) were starved by resuspension in MES buffer for 2 hours. A portion was stimulated by the addition of glucose (25 mM) and samples were taken over 45 min. Reactions were halted with MeOH:$CHCl_3$ and radioactivity was extracted *via* the Bligh and Dyer (1959) method. Extracts were eluted from HPLC anion exchange columns and fractions (2 ml) were counted in Optiphase 'Safe' (8 ml). A:- Stimulated, B:- Non-Stimulated.

slight modification of the assay methodology. Concern was expressed about the proportion of radioactivity derived from the tertiary supernatant that was analysed with the cell extract. A repeat experiment was performed, but the stimulated and non-stimulated cells were harvested by centrifugation before reactions were halted by resuspension in methanol:chloroform (2:1 v/v). In this way a separate tertiary supernatant was recovered from the turnover experiment samples for independent analysis. The previously seen increase in GPI may simply have been a result of cell metabolism as PI was deacylated and lost to the medium, and not glucose-stimulated turnover within the cell. Isolation of the reaction buffer removed this factor from the final result.

The elution of extracts from both stimulated and non-stimulated cells in the absence of the tertiary supernatant from HPLC columns gave a very different result (Fig. 3.24). All four extracts showed the presence of an inositol peak, which decreased in magnitude slightly when glucose-stimulated, but remained constant in the non-stimulated cells. In the stimulated samples, an area of slightly elevated counts was present in the vicinity of GPI and IP_1. No distinct peaks were discernible and the levels did not seem to alter with time. It appeared as if the majority of radioactivity detected in the previous experiment had been derived from the tertiary supernatants, and there was no evidence for inositol-phospholipid turnover within the cell. The analysis of the tertiary supernatants confirmed the

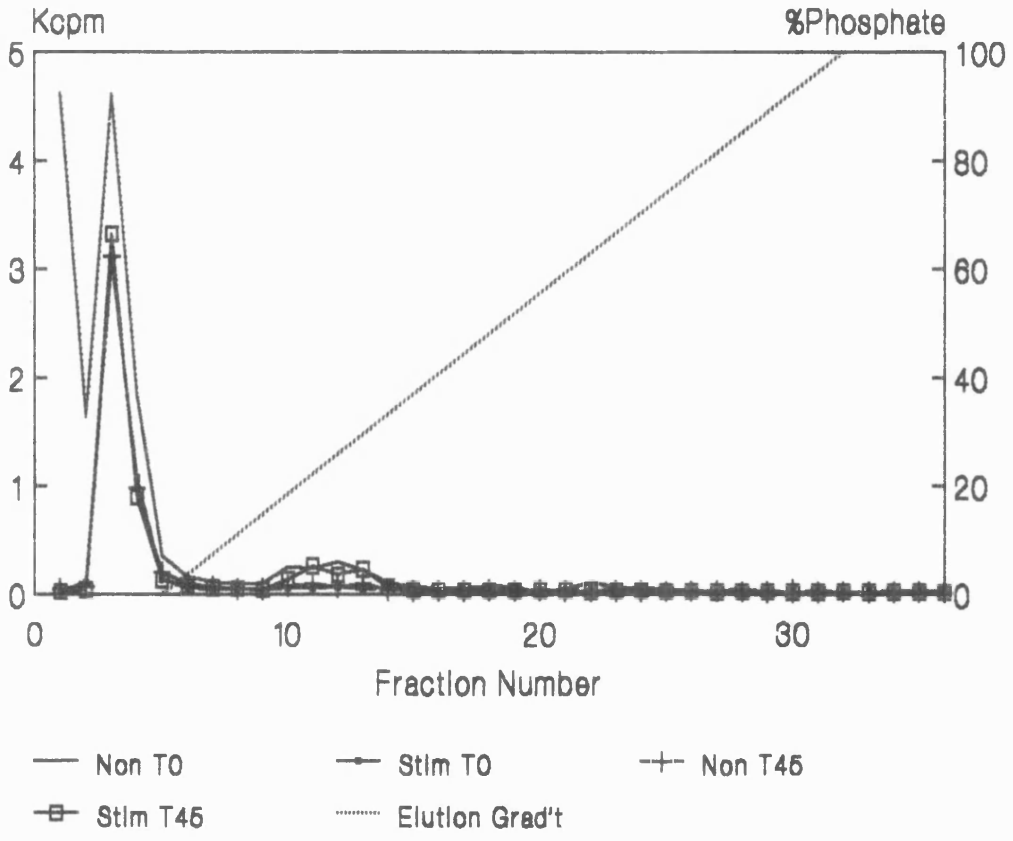


Fig. 3.24. Elution of Cell Extracts from Glucose Induced PIP_2 Turnover Experiments without Tertiary Supernatant

Cells were labelled and treated in the same manner as described for Fig. 3.23. Prior to extraction of radioactivity, the cells were harvested by centrifugation and the tertiary supernatant removed. Extracts were eluted from a 10 µm SAX HPLC column using a linear gradient of 0-100% $NH_4H_2PO_4$ (1.4 M). Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for liquid scintillation counting.

observation (Fig. 3.25) - inositol and GPI peaks were detected in both the extracts of stimulated and non-stimulated cells. In the stimulated cells, a decrease in inositol concentration was coupled with an increase in GPI concentration with time. The opposite effect was seen in the non-stimulated cells. Although the previous experiment had shown an increase in GPI in both sets of extracts, the overall inference was that GPI production was a response to glucose stimulation (in starved cells) with an accompanying decrease in inositol.

Considering the aqueous-methanol extracts (Fig. 3.24), the absence of peaks was thought to be due to phosphatase degradation of potentially present inositol phosphates prior to the assay. An experiment was therefore performed in which the period of incubation in buffer was reduced to 1 h, to investigate the possibility that prolonged exposure to the starvation stress prevented the cells from recovering to a viable condition when stimulated. A reduction in the time that the cells were subjected to this metabolic strain was hoped to maintain a condition in which they could initiate inositol phospholipid turnover when stimulated. Cells were harvested after a 20 min stimulation with glucose. If turnover had occurred, and was accompanied by phosphatase activity, then a shorter incubation period would reveal inositol phosphates that had not yet been degraded. The results are summarized in Fig. 3.26. Analysis of the buffer after removal of the cells showed an inositol peak and an area of radioactivity slightly elevated above background

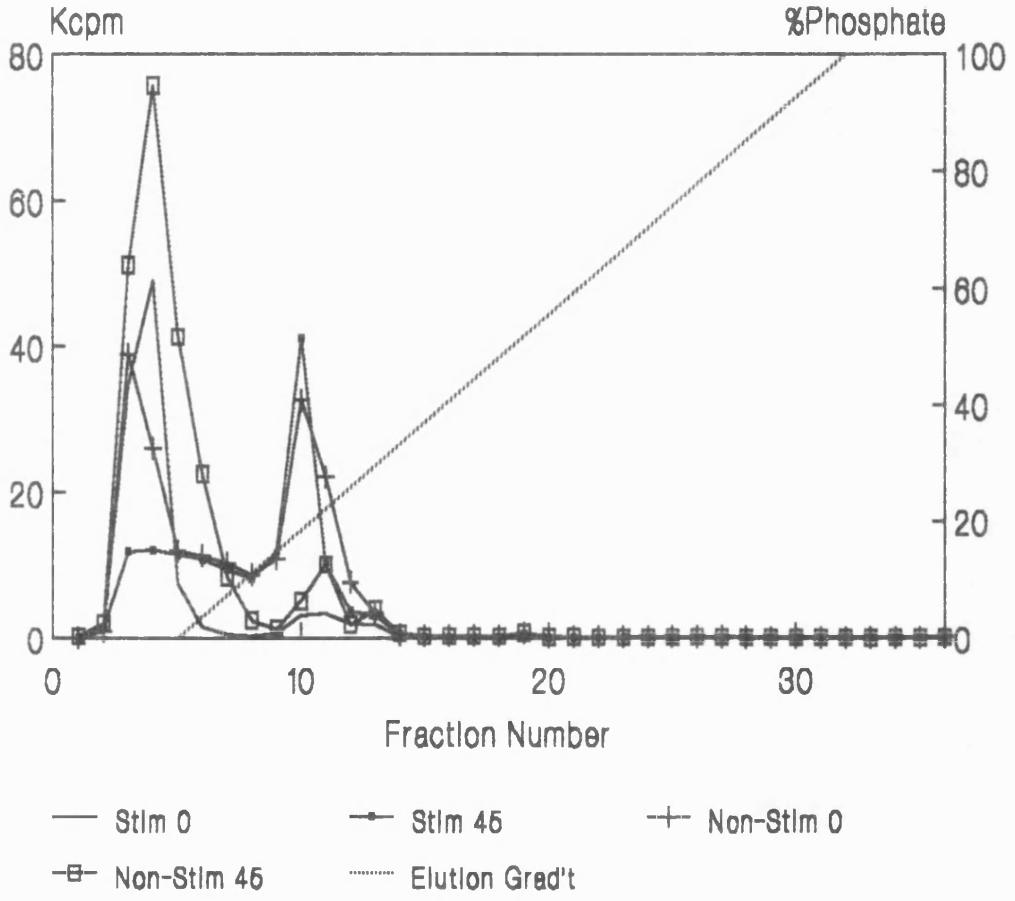


Fig. 3.25. Analysis of Tertiary Supernatants from Glucose Induced Turnover of PIP_2 Experiment

The tertiary supernatants from the experiment illustrated in Fig. 3.24 were eluted from a 10 µm SAX HPLC column using a linear gradient of $NH_4H_2PO_4$ (1.4 M, 0-100%). Fractions (2 ml) were collected and mixed with Optiphase 'Safe' for detection by liquid scintillation. Inositol and GPI peaks were detected in all samples.

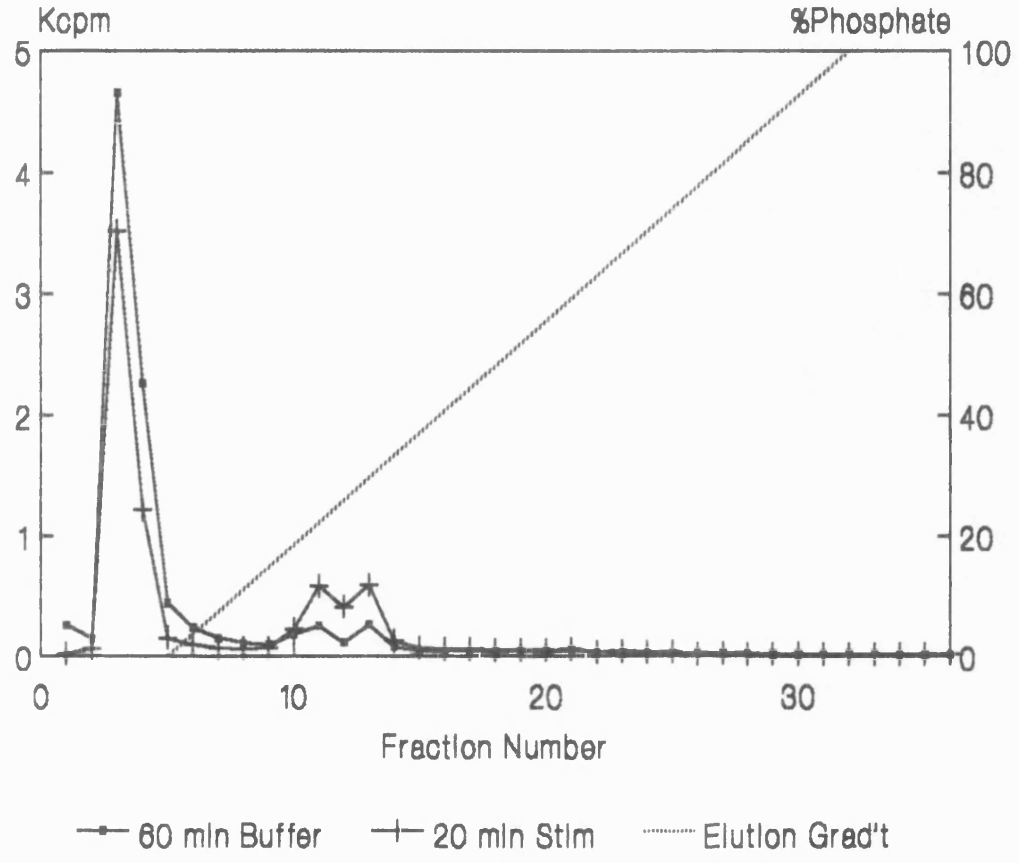


Fig. 3.26. Analysis of Samples from Glucose Induced Turnover Experiment with Reduced Incubation Periods

Cells were labelled and treated according to the details given in the text. Stimulated cells were harvested after 20 min. Reactions were halted, and radioactivity was extracted following the removal of the tertiary supernatant. The starvation buffer and extract were eluted from a 10 µm SAX HPLC column using a linear gradient of $NH_4H_2PO_4$ (1.4 M, 0-100%). Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for liquid scintillation counting.

level in the position of GPI and IP_1. The cell extract gave a profile almost identical to that seen in the previous experiment - a large inositol peak with a much smaller peak comprised of GPI and IP_1. No higher inositol phosphates were detected, and the levels of GPI and IP_1 were not significantly greater than those previously recorded. The theory of phosphatase degradation did not obtain, and no evidence of glucose-induced turnover was seen. The similarity of the profiles from the buffer and cell extract possibly suggested that the eluted peaks were caused by degradation of an inositol-containing metabolite that was present inside the cell, but was deposited outside as well. No independent evidence was available to confirm or refute this theory.

The analysis of the tertiary supernatants from the non-stimulated cells showed the presence of peaks corresponding to inositol and GPI as expected but the supernatants from the stimulated cells produced a novel profile with peaks in the positions of inositol, GPI and IP_1 (Fig. 3.27). A monophosphate peak had not been previously detected in the tertiary supernatant, but was present in both the zero time and 45 min samples. Both the GPI and IP_1 peaks showed a small increase in concentration over the 20 min incubation period, but little could be concluded about the behaviour of inositol because of the ill-formed peak in the second extract. Close examination of the elution profiles suggested the presence of a fourth peak at fraction 19. It was first thought that this may have indicated the presence of a

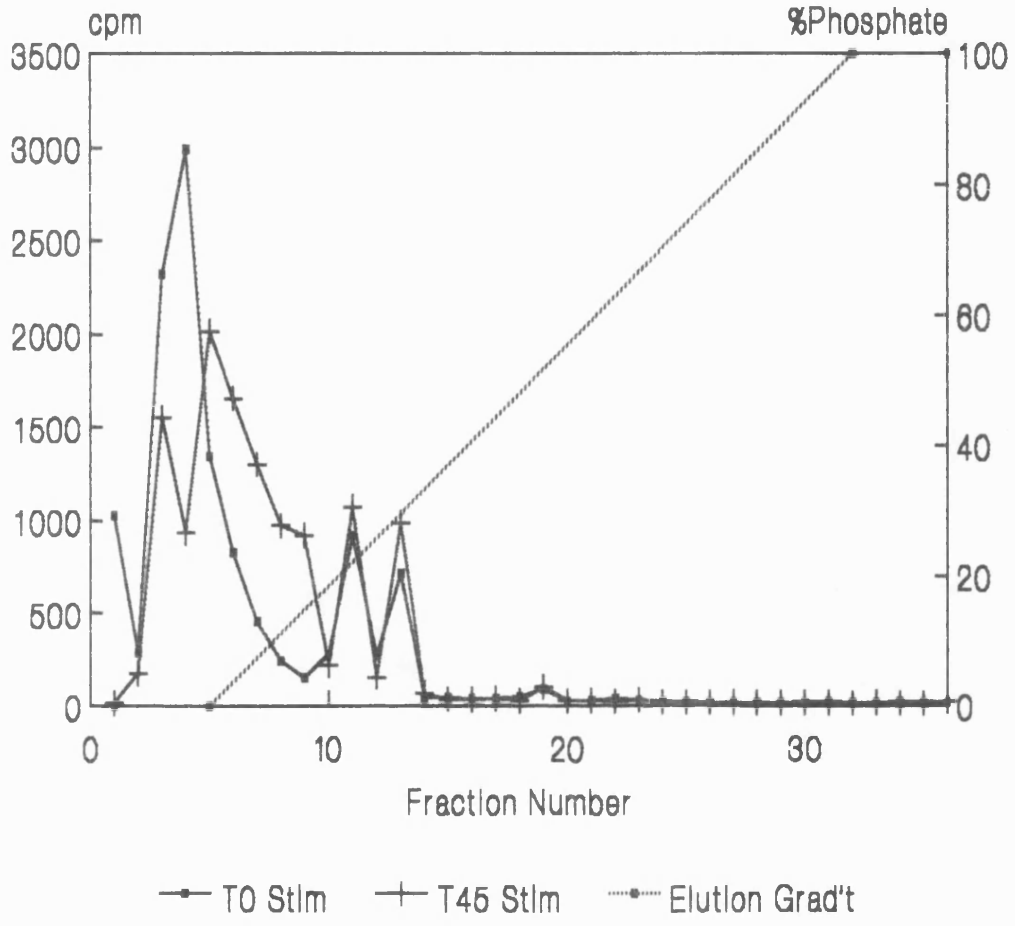


Fig. 3.27. Analysis of Tertiary Supernatants from Stimulated Cells - Glucose Induced PIP_2 Turnover (Short Incubations)
Tertiary supernatants were removed from cell samples prior to extraction of radioactivity (see Fig. 3.24, 3.25). The supernatants were eluted from a 10 µm SAX HPLC column using an increasing linear gradient of $NH_4H_2PO_4$ (1.4 M, 0-100%). Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation counting

trisphosphate, but standard Ins(1,4,5)P_3 eluted in fractions 22-24. The peak could not immediately be identified, but this result suggested that further metabolites could be isolated and separated following modification of the assay conditions.

Although it had become standard procedure to monitor the growth of cells during the 24 hour incubation period, increases in density were recorded, but the actual phase of growth referred to by the readings was not known. A growth curve of strain MC3 in the assay conditions was therefore obtained (Fig. 3.28). The result showed that cells grown in the conditions dictated by the experimental procedure could take up to 40 h to achieve stationary phase. It was possible that previous experiments had all been performed with cells still in the exponential phase of growth. These cells may not have responded in the same way to a glucose stimulus because they were still actively proliferating and were still utilizing glucose present in the growth medium. Conditions would not have been limiting and the stimulus would not have induced inositol phospholipid turnover. An experiment was therefore performed in which the cells were incubated with the radioactive tracer inositol for 48 hours before harvesting by centrifugation. This was to ensure that the stationary phase of growth had been reached. Cells were resuspended in fresh buffer and one portion was stimulated with glucose as previously described. Samples from both portions were taken at time zero and after 45 min, and treated as above.

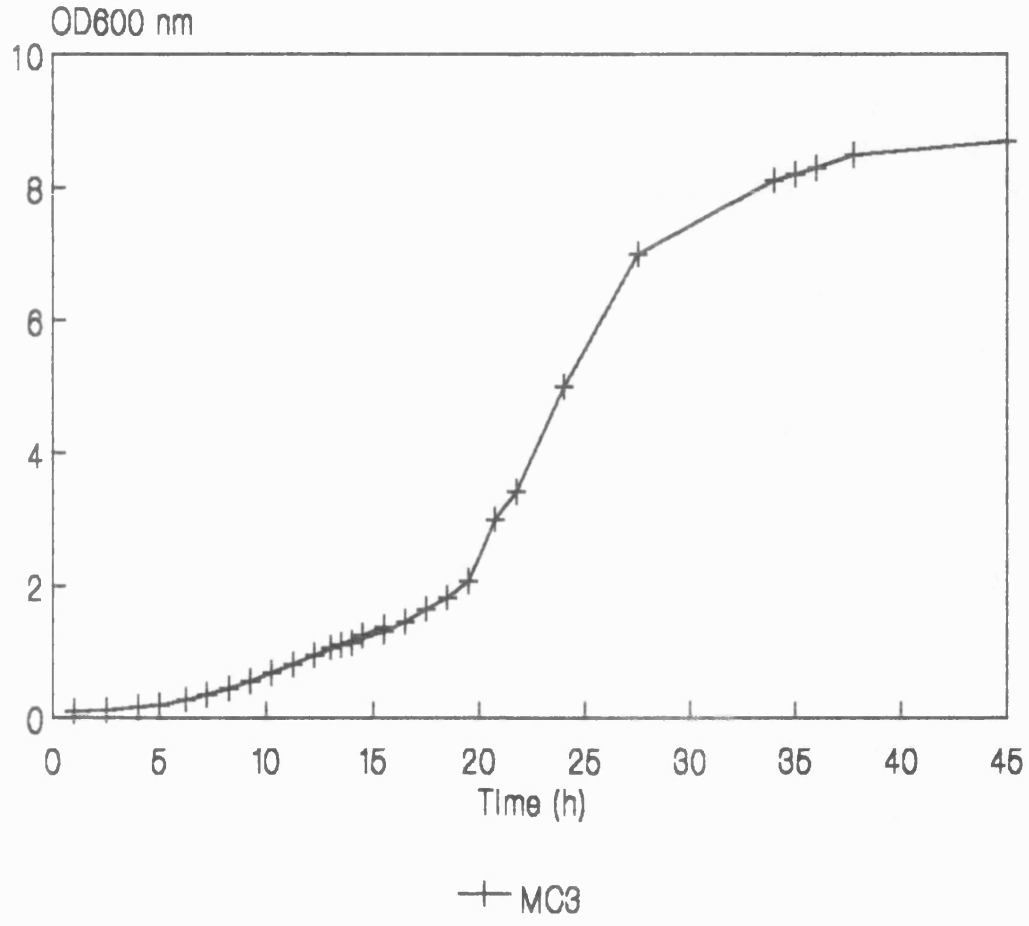


Fig. 3.28. Growth Curve of Strain MC3 in Yeast Nitrogen Base Medium

Yeast Nitrogen Base medium (400 ml) was inoculated with strain MC3 and incubated at 30 ^{o}C with shaking (200 RPM). Samples (2 x 1 ml) were removed during the incubation period, and growth was monitored by measuring the optical density at 600 nm. The mean optical density reading was plotted against time.

Analysis of the aqueous extracts from the Bligh and Dyer (1959) treatment showed exactly the same result obtained from earlier experiments - the presence of an inositol peak in all extracts and very small peaks in the GPI/IP1 retention areas (e.g. Fig. 3.24, 3.26). There was no evidence to suggest that the extended incubation had had any effect in stimulating inositol phospholipid turnover.

Analysis of the tertiary supernatants did however show the presence of peaks. The glucose-stimulated extracts both showed peaks that corresponded to inositol, GPI, IP1 and the previously seen peak in fractions 19-21 (Fig. 3.29). Following stimulation, the inositol peak showed a decrease in magnitude whereas the other three peaks all showed an increase. A similar profile was produced by the non-stimulated cells (Fig. 3.30). The same peaks were detected and in the case of inositol, GPI and the IP_1 peaks, the same behaviour was recorded. The most noticeable difference between the two results was that in the fraction 19 peak of the non-stimulated cells, no increase was observed. Profiles of this type had not previously been detected in these experiments. It was proposed however that the observed peaks did not actually represent the detection of myo-inositol phosphates that had been released to the medium, but the products of deacylated inositol-containing phospholipids (Hawkins, P., 1992; pers. comm.). The presence of three distinct peaks suggested that the compounds detected were GPI, GPIP and $GPIP_2$ - water-soluble products of the phosphoinositides, formed following

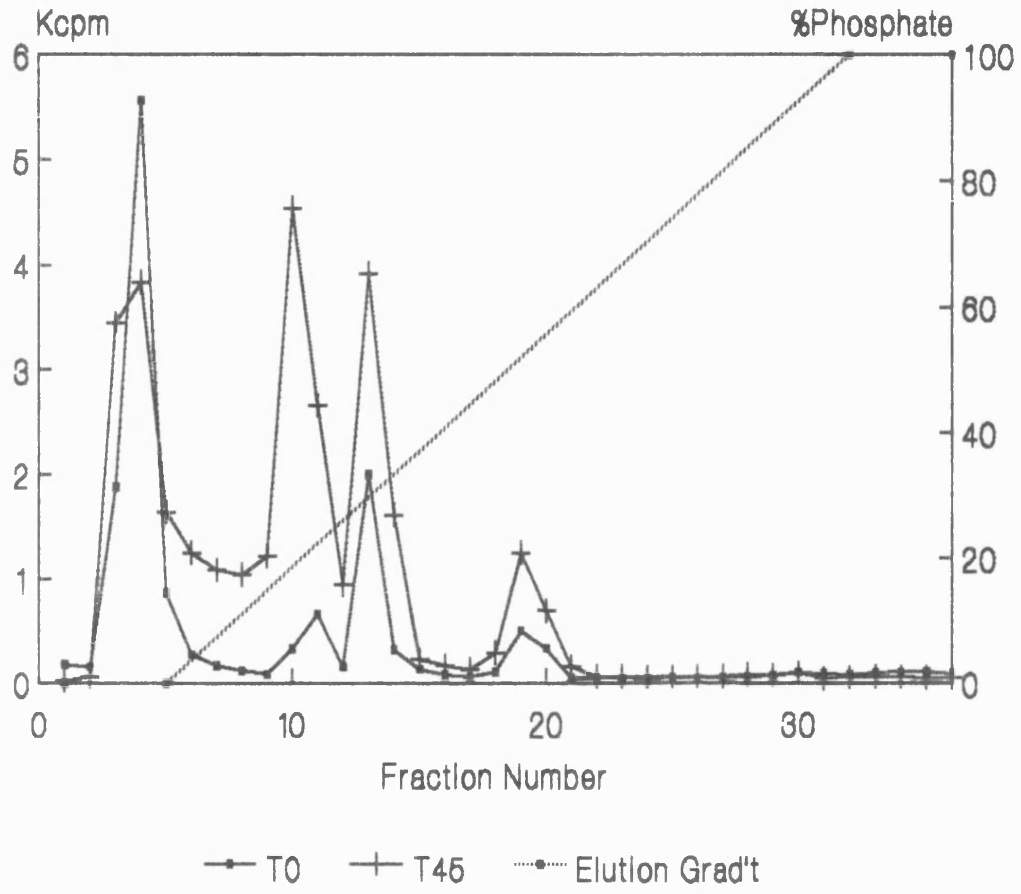


Fig. 3.29. Analysis of Tertiary Supernatants from Stimulated Cells After Extended Incubation Period

Cells treated as previously described were incubated with [^{3}H]-inositol (20 µCi ml^{-1}) for 48 hours. Stimulation with glucose (25 mM) was performed after 2 hours incubation in MES buffer. The tertiary supernatants were eluted from a 10 µm SAX HPLC column and fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation counting.

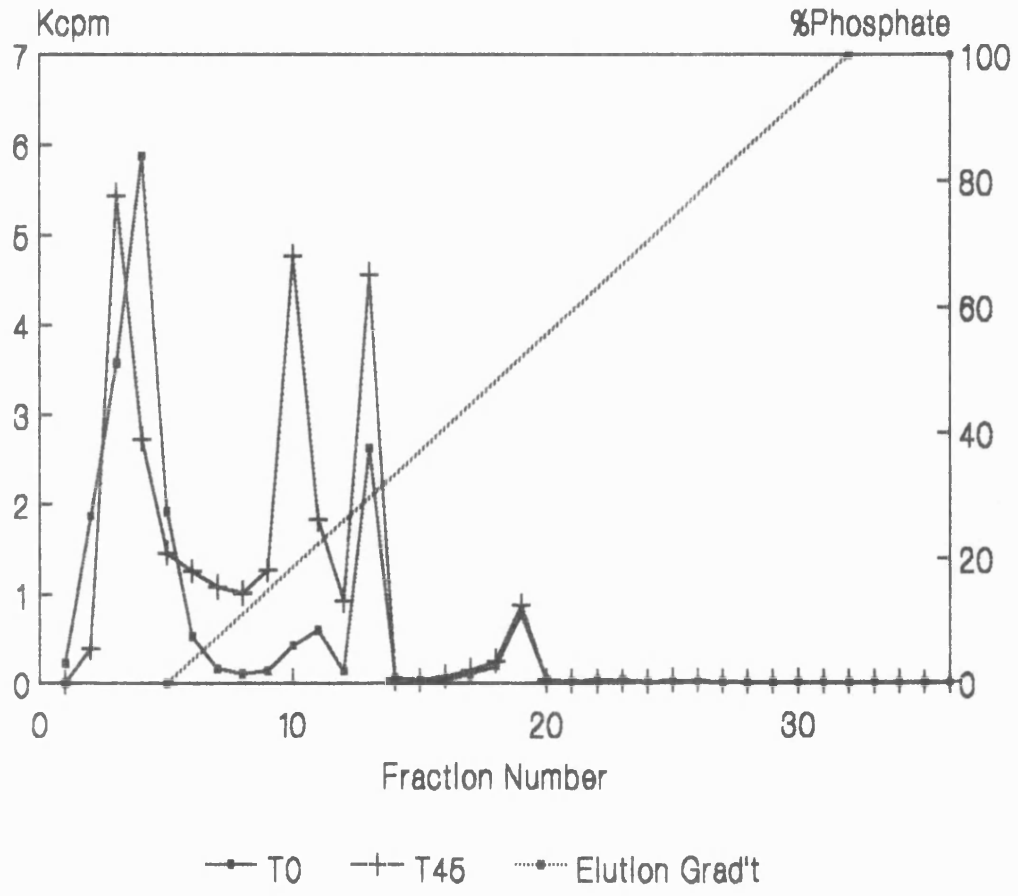


Fig. 3.30. Analysis of Tertiary Supernatants from Non-Stimulated Cells after Extended Incubation

Cells treated as previously described were incubated with [^{3}H]-inositol (20 µCi ml^{-1}) for 48 hours, then resuspended in MES buffer for a further 2 hours. Following resuspension in fresh MES, tertiary supernatants of time zero and 60 min samples were eluted from a 10 µm SAX HPLC column. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation counting.

phospholipase A treatment. The result indicated an increase in concentration of GPI and 'GPIP' irrespective of glucose stimulus, although the '$GPIP_2$' peak only increased in the presence of glucose. The compounds could not be positively identified because genuine standards were not available but the release of phosphoinositide derived by-products to the medium helped to explain the results obtained. The increase in '$GPIP_2$' recorded in the last experiment was the only available evidence for turnover of PIP_2 in response to glucose as described by Kaibuchi *et al.* (1986). The increase in GPI and 'GPIP' in both stimulated and non-stimulated cells did not support this evidence though, it was not known if this was simply a result of resuspension in fresh buffer or just a previously unseen result.

It was not known if the phospholipases that degraded the inositol phospholipids were stimulated by glucose or whether the enzyme activity was continuous, irrespective of stimulation.

The overall conclusion from the experiments performed was that the observations of Kaibuchi *et al.* (1986) could not be repeated. No evidence was provided for the production of inositol trisphosphate throughout the investigation and hence no evidence was obtained in favour of the presence of a mammalian-like inositol phospholipid turnover system.

A number of experiments were also performed to enhance the possible turnover of inositol phospholipids and improve

radioactivity recovery from the aqueous extracts. Having determined that a 48 h incubation period was necessary for the cells to reach stationary phase of growth, the necessity of an incubation in MES buffer was questioned. Cells at stationary phase may already find the conditions limiting and be unable to metabolize further without some form of stimulus. Cells were therefore treated as normal until after the incubation with [^{3}H]-inositol. The cells were then directly stimulated with glucose at a final concentration of 25 mM and samples were taken at time zero and 60 min. Analysis of the aqueous extracts showed the presence of an inositol peak and very small peaks in the GPI/IP_1 area. The same profile had been seen in almost all of the aqueous extract analyses throughout the investigation. The omission of the MES buffer incubation stage had had no effect on the result. It was considered that glucose was not a sufficiently specific stimulus for the putative transduction system, or did not provide the necessary requirements to initiate turnover. A repeat experiment was therefore performed in which a 60 min incubation in MES buffer was included, but the subsequent stimulus was provided by resuspending the cells in fresh YEPD medium - this was hoped to include a nutrient stimulus as well as a glucose stimulus. Samples were taken at time zero and 60 min but the analysis of aqueous extracts showed exactly the same profiles as above. The use of YEPD medium as a stimulus had not shown any improvement in inositol phosphate production.

At a later date, the tertiary supernatants were analysed having been stored at -20 ^{o}C for a number of weeks. The elution of supernatants from the experiment without MES buffer incubation showed the presence of peaks corresponding to inositol, GPI, 'GPIP' and 'GPIP$_2$' (Fig. 3.31). The profiles were dominated by huge inositol peaks which effectively masked all other peaks, and doubled in concentration during the experiment. Although a very small increase in 'GPIP$_2$' can be seen, the GPI and 'GPIP' peaks both show a marked decrease over the 60 min period. This experiment was not repeated because the main area of interest when it was performed was the contents of the aqueous phase, but in contrast to other tertiary supernatant analyses the exclusion of the buffer incubation stage has promoted degradation of some of the peaks. The previously obtained profiles (Fig. 3.29) showed universal increases when stimulated. Analysis of the supernatants from cells stimulated by resuspension in YEPD (after an incubation in MES buffer) did show increases in peaks corresponding to inositol, GPI, 'GPIP' and 'GPIP$_2$' over a 60 min period (Fig. 3.32) and supported observations made earlier. This experiment was not repeated for the reasons described above, but there is *prima facie* evidence to suggest that all the potentially phosphoinositide-derived peaks increase in concentration following glucose stimulation, and that removal of the MES incubation stage greatly affects this activity. It would appear that the detection of radioactive compounds in the reaction medium can provide more information pertaining to glucose induced turnover of

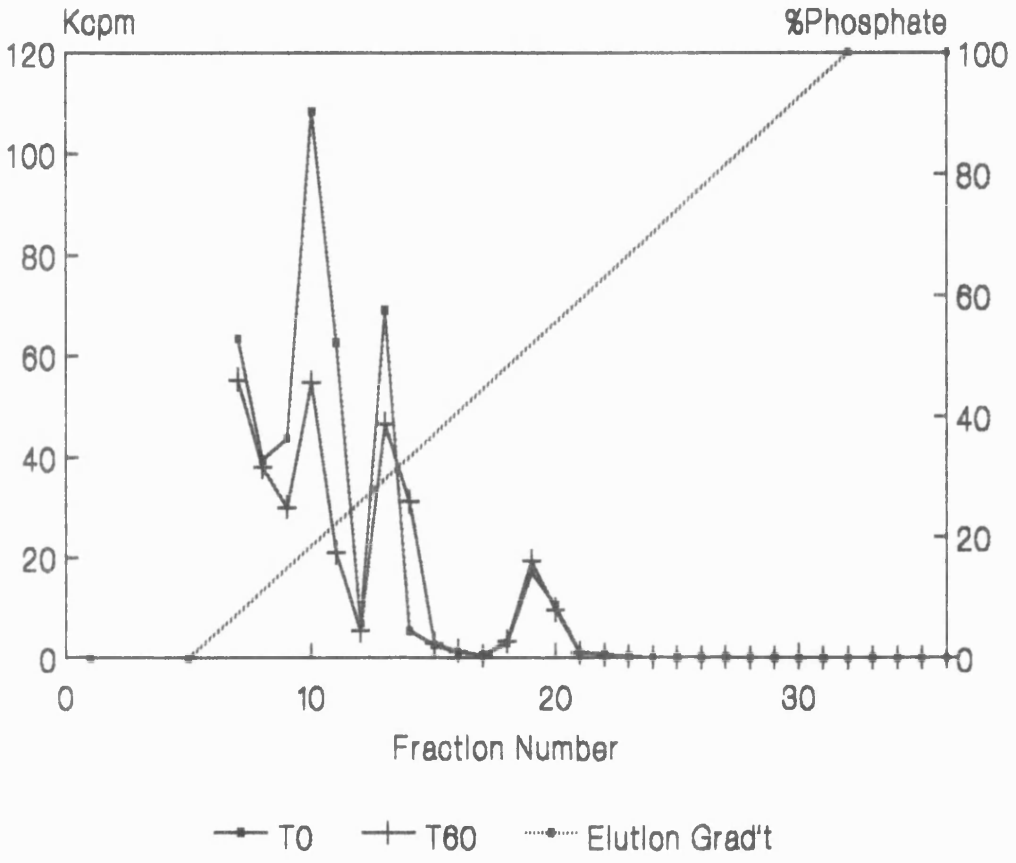


Fig. 3.31. **Analysis of Tertiary Supernatants of Cells Stimulated Without a Buffer Incubation Stage**

A sample (2 ml) of a mid-exponential culture of MC3 was incubated for 48 hours at 30 ^{o}C in YNB + [^{3}H]-inositol (20 µCi ml^{-1}). The cells were stimulated by the direct addition of glucose (25 mM final concentration) and samples (1 ml) were taken at time zero and 60 min. The supernatants were eluted from a 10 µm SAX HPLC column and collected fractions (2 ml) were mixed with Optiphase 'Safe' (8 ml) for liquid scintillation counting.

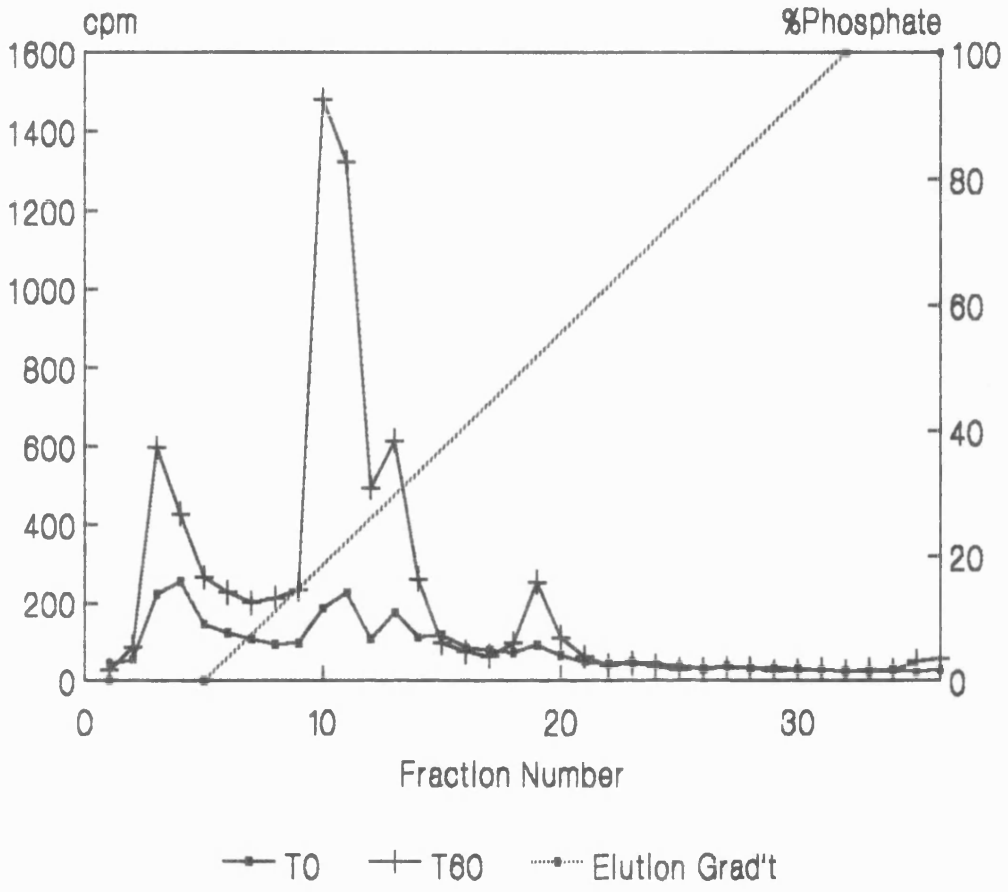


Fig. 3.32. Analysis of Tertiary Supernatants from Cells Stimulated by Resuspension in YEPD

A glucose-induced PIP_2 turnover experiment was performed as described in the text except that stimulation was by resuspension in YEPD (2 ml). Samples (1 ml) were taken at time zero and 60 min. Supernatants were eluted from a 10 µm SAX HPLC column and collected fractions (2 ml) were mixed with Optiphase 'Safe' (8 ml) for liquid scintillation counting.

inositol containing phsospholipids than analysis of aqueous extracts. Although GPI and IP_1 peaks were regularly detected in the cytosol following stimulation, higher inositol phosphates (IP_2) were only detected in early experiments with low activity levels of ^{14}C-labelled inositol. Inositol bisphosphate was not detected at higher labelling concentrations but the very low levels of the cytosolic inositol phosphates detected and the use of a less stable tracer may have exacerbated recovery.

3.8 Assay of IP_3 Phosphatase Activity

Perhaps the inability to detect an IP_3 peak in a yeast cell preparation was due to the activity of an IP_3 degrading enzyme (e.g. IP_3 phosphatase), which removed trisphosphate before it could be isolated or the reactions halted. Experiments were therefore planned to demonstrate the ability of a cell preparation to degrade exogenously added tritiated IP_3. Once the degradation had been monitored, suitable inhibitors could then be included in the assay to either slow or halt the destruction. If the activity of the phosphatase could then be inhibited *in vitro*, it would facilitate recovery of the trisphosphate produced as a result of agonist-stimulation.

In mammalian cells, IP_3 phosphatase is described as a '2C phosphatase' as it is activity-dependent on the presence of Mg^{2+} ions (Cohen, 1989). Cohen *et al.* (1989) also reported that there were remarkable similarities between yeast and

mammalian protein phosphatases with reference to type 1, type 2A and type 2C enzymes, so it was thought that inhibitors of mammalian IP_3 phosphatase might also be effective against the yeast enzyme. Rana *et al.* (1986) demonstrated that in rat pancreatic islets, the hydrolysis of IP_3 was completely inhibited by omitting magnesium from the enzyme reaction mixture or by adding 10 mM EDTA to chelate magnesium. Another inhibitor was suggested by Seyfred *et al.* (1984) who noted that spermine (2.0 mM) inhibited IP_3 phosphatase activity by 50% in rat liver plasma membranes, and Hansen *et al.* (1987) demonstrated that Ca^{2+} in the micromolar range can reduce phosphatase activity in rat brain. The commonly used protein phosphatase inhibitor, okadaic acid was found to be ineffective against IP_3 phosphatase in mammalian cells (Haystead *et al.*, 1989; Bialojan and Takai, 1988) and was therefore not considered for investigation in yeast. Other potential inhibitors such as ATP and glycolytic intermediates have been recorded (see Shears, 1989) but regulating their concentration *in vivo* would be difficult to achieve, so these were not immediately considered for use. They were however available for future investigations if required.

In the initial IP_3 phosphatase activity assays, (Materials and Methods 2.10.3) no degradation of IP_3 was recorded in duplicate experiments. Although the distribution of IP_3 phosphatase between membrane and cytosol was not known, it was thought that the inclusion of the whole cell homogenate in the assay would facilitate detection of activity. This

had not been shown however and it was decided to repeat the assay in a different buffer in case the high concentration of phosphate was interfering with the enzyme activity. The assay was performed as previously described, but the sonicated cells were resuspended in Tris/HCL buffer. No degradation of the exogenously added ^{3}H-IP_3, was recorded between pH 7.0 - 7.4 and phosphatase activity could not be demonstrated. To examine the possibility that the sonication treatment was not adequately breaking the cells, a series of phosphatase assays were performed using cell extracts produced using the Braun homogeniser (see Materials and Methods 2.18). The cell pellet was suspended in 5 ml of the relevant buffer for breaking, then the beads were washed in fresh buffer until 30 ml had been recovered for the assay. This also failed to demonstrate a decrease in trisphosphate radioactivity counts. It was not possible to show the degradation of exogenously added tritiated Ins(1,4,5)P_3 using a crude yeast cell preparation. Three possible explanations were considered for the lack of activity:- (1) IP_3 phosphatase was not present, (2) the enzyme was active, but the assay was not sensitive enough or (3) the enzyme was inactive or had been destroyed.

Believing the enzyme to be active, an improved buffer was prepared following a review of phosphatase assays in mammalian cells (Seyfred *et al.*, 1984; Hansen *et al.*, 1987) which included Mg^{2+} ions and 1 mM dithiothreitol (Connolly *et al.*, 1987; Hoer *et al.*, 1990; Fowler and Brännström, 1990). Further assays were then performed using Braun

homogenisation as the method of cell breakage. The homogeniser appeared to give a higher degree of cell breakage, but this was not quantified. Cells were broken in the new buffer (Tris/HCL pH 7.0, 50 mM; $MgCl_2$, 3 mM; dithiothreitol (DTT), 1 mM) and the assay was performed as described previously. The radioactivity collected during elution of the IP_2 'window' of the Dowex anion exchange column was also counted, so that any decrease in the concentration of IP_3 could be correlated with a potential increase in IP_2 concentration. Although the initial experiment appeared to show a reduction in the level of IP_3, the result was misleading. The zero time sample was not treated in the same manner as the subsequent samples (boiling for 2 min etc) and was not directly comparable. The repeat experiment in which all the samples were treated identically showed no degradation of the trisphosphate and no increase in the IP_2 levels.

An alternative assay for phosphatase enzymes was provided by Hanson (1991) who described the activity of phosphoinositides and the inositol phosphates in *Neurospora crassa*. Harvested cells were resuspended in HEPES buffer (1.5 mM, pH 7.0) and homogenised. The broken cell homogenate was centrifuged at 2000 x *g* for 5 min and the supernatant was centrifuged at 40,000 x *g* for 30 min. The pellet was then resuspended in 1 ml of the new phosphatase buffer and used as the enzyme source. In the first experiment the enzyme preparation was incubated with ^3H-IP_3 (0.04 µCi) for 20 min at 25 ^oC and reactions were terminated by the

addition of 1 ml ice-cold TCA (15% v/w). The mixture was incubated on ice for 10 min, then washed with diethyl-ether (5 x 2 ml). Analysis of samples by Dowex anion exchange chromatography showed no significant variation in IP_3 radioactivity. The IP_2 peak however appeared to almost double, indicating that some metabolism had occurred (Fig. 3.33). To investigate further, the incubation period was increased to 2 hours in a second experiment. The immediate observation was that after 40 min, the IP_3 peak had disappeared (Fig. 3.34). There was however no concomitant increase in the IP_2 peak or lower inositol phosphates. If the IP_3 peak was fully degraded, it was not known to where the radioactivity had been redistributed. It is also important to note that the samples obtained at T0 min and T20 min were injected onto the column without a filtration clean-up step. All samples subsequent to this were filtered using a Sartorius Minisart NM2 micro filter following centrifugation to ensure all solid material had been removed. The filtration step may have affected the sample significantly and had somehow sequestered inositol phosphates.

Filtered and unfiltered ^3H-IP_3 samples were then analyzed by HPLC. Boiled and non-boiled samples were also run to assess the possibility of activity loss by these methods. No major loss of radioactivity was recorded in either case (data not shown) confirming the previous result.

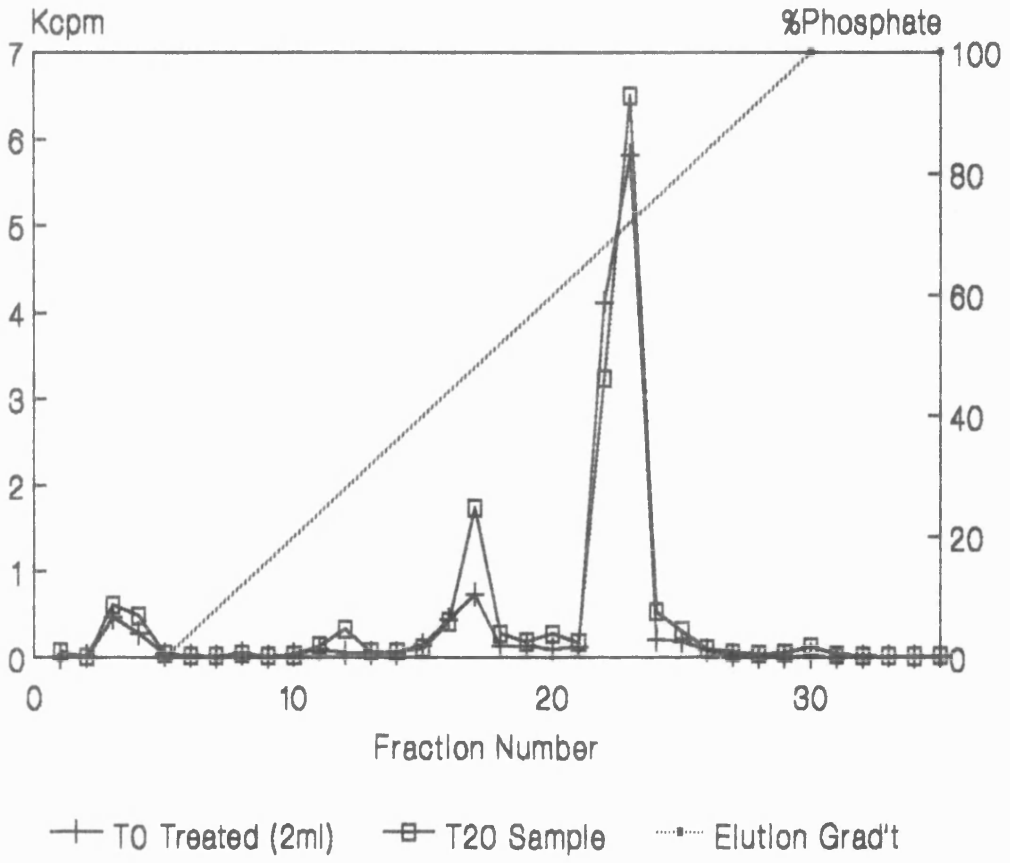


Fig. 3.33. Hanson (1991) Style IP_3 Phosphatase Assay
Strain MC3 (400 ml) was grown to late-exponential phase in YEPD. The enzyme source was prepared as described and resuspended in phosphatase buffer. This was then mixed with ^3H-IP_3 and incubated for 20 min at 25 ^oC with shaking (120 RPM). Samples (1ml) were halted at 0 and 20 min using ice-cold TCA (15% w/v) and were analysed by elution from a 10 µm SAX HPLC column. Fractions (2ml) were collected and mixed with Optiphase 'Safe' for detection by liquid scintillation counting.

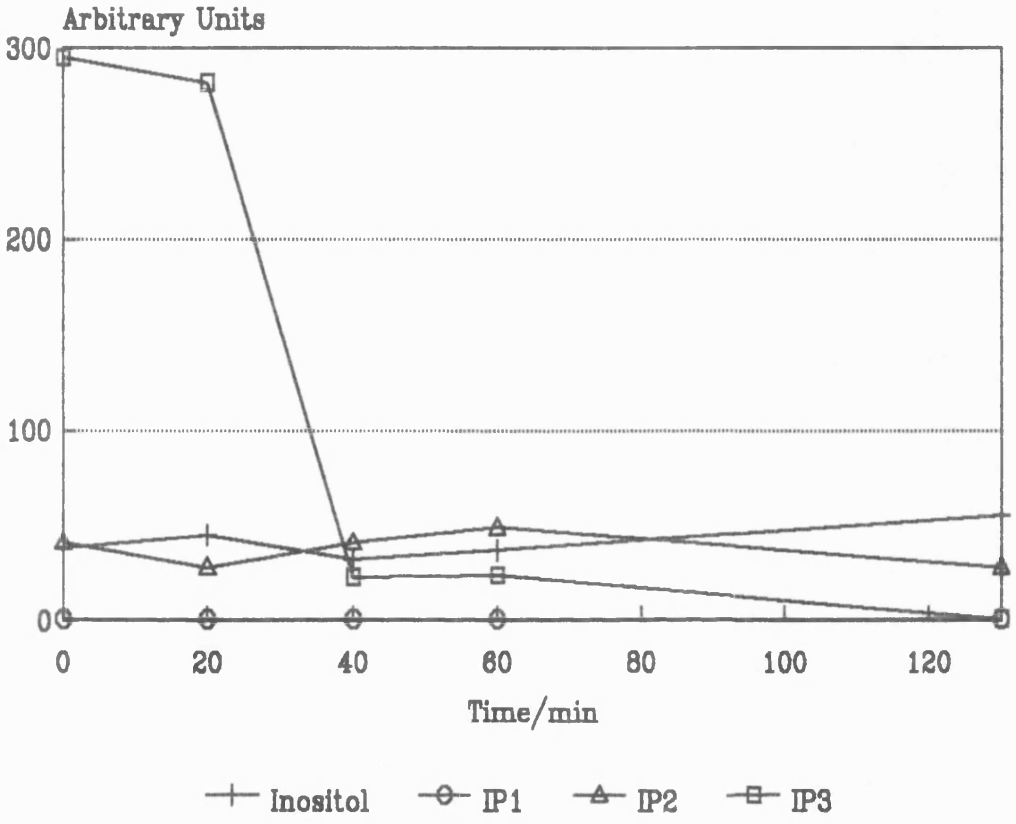


Fig. 3.34. Two Hour IP_3 Phosphatase Assay (Hanson, 1991)
A mid-exponential culture of MC3 was harvested and washed, cells were broken in the Braun homogeniser and an enzyme source was prepared as described (Materials and Methods 2.18). The cell extract was resuspended in 2 ml of IP_3 phosphatase buffer (see text) and incubated at 25 ^{o}C for 2 h with 0.12 µCi of ^{3}H-IP_3 in buffer (3 ml). Samples (1 ml) were taken during the incubation and reactions were halted by the addition of ice-cold TCA (15% w/v). Samples were analysed by HPLC and area under the curve values calculated.

To confirm the activity observed in the previous IP_3 phosphatase assay and remove confusion caused by the use of filters in sample preparation, the Hanson (1991) method was investigated more fully. The methodology was similar to the preceding experiment but the enzyme preparation was resuspended in 30 mM HEPES (pH 7.0). The incubation conditions were exactly the same, but reactions were halted by the addition of an equal volume of ice-cold pyridine solution (ethyl alcohol: diethyl ether: pyridine, 15:5:1 v/v/v after Hanson, 1991). Anion exchange chromatography was performed in 10 cm Dowex resin columns connected to the HPLC, allowing a linear gradient to be applied. The analysis was performed in this manner such that samples could be examined with the minimum of preparation, avoiding any procedures which may have affected the radioactivity content. The result (Fig. 3.35) shows a gradual decrease in the height of the IP_3 peak with time. The relatively slow turnover may be a result of the omission of $MgCl_2$ from the incubation buffer. This result does not show a coincident increase in any other peak or area of the profile. The expected result was an increase in the peak height of IP_2 corresponding to a decrease in IP_3. The discernible peaks of IP_3, IP_2 and IP_1 all seem to degrade and it is only in the T120 min sample that an increase is recorded for the level of free inositol. Although the degradation of exogenously added IP_3 was confirmed in this experiment, no evidence was provided for the existence of a degradative pathway for the inositol phosphates in yeast.

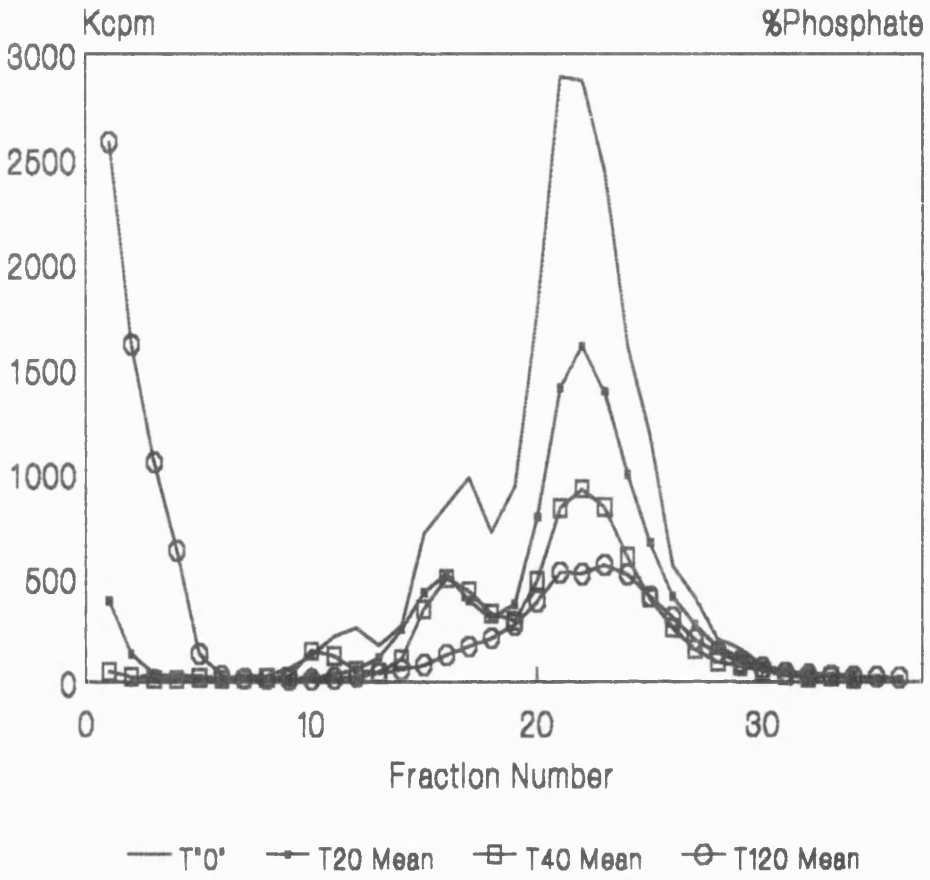


Fig. 3.35. Extended IP_3 Phosphatase Assay (Hanson, 1991) The methodology was as described for Fig. 3.34. Samples (1 ml) were removed during this time. Reactions were halted by the addition of an equal volume of pyridine solution (see text) and samples were analysed by elution from a 10 cm Dowex anion exchange column connected to the HPLC. A linear gradient of $NH_4H_2PO_4$ (0-100%) was used and fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation spectrometry.

Experiments were then performed exactly as described by Hanson (1991). Details are given in Materials and Methods 2.18 and 2.19. The enzyme preparation was resuspended in 1.5 ml of 30 mM HEPES pH 7.0/3 mM $MgCl_2$. Duplicate experiments gave essentially identical results, showing a progressive decrease in the height of the IP_3 peak with time (Fig. 3.36). There was a marked reduction in the trisphosphate peak during the first few seconds of the assay after which the activity slowed down considerably. The inclusion of Mg^{2+} ions in the incubation buffer may have speeded up the reaction rate but had little effect on the formation of lower inositol phosphates in response to the degradation of IP_3. As in the previous assay, all three component peaks were gradually degraded with only a very transient increase in the magnitude of the IP_1 peak. There was no increase in the size of the IP_2 peak throughout the assay, the area under the curve showed a continual reduction with a similar profile to the IP_3 result. Perhaps the reduction of IP_3 with no corresponding increase in IP_2 or IP_1 was an aspect of sequestration of radioactivity rather than genuine enzymatic activity. Any remaining pellet material from the assay samples was subjected to a Bligh and Dyer (1959) extraction in an attempt to recover any "bound" radioactivity from the cell material. Both the aqueous and organic phases produced only background counts however and provided no further information.

To examine further the possibility of genuine enzymatic activity being responsible for the reduction of the

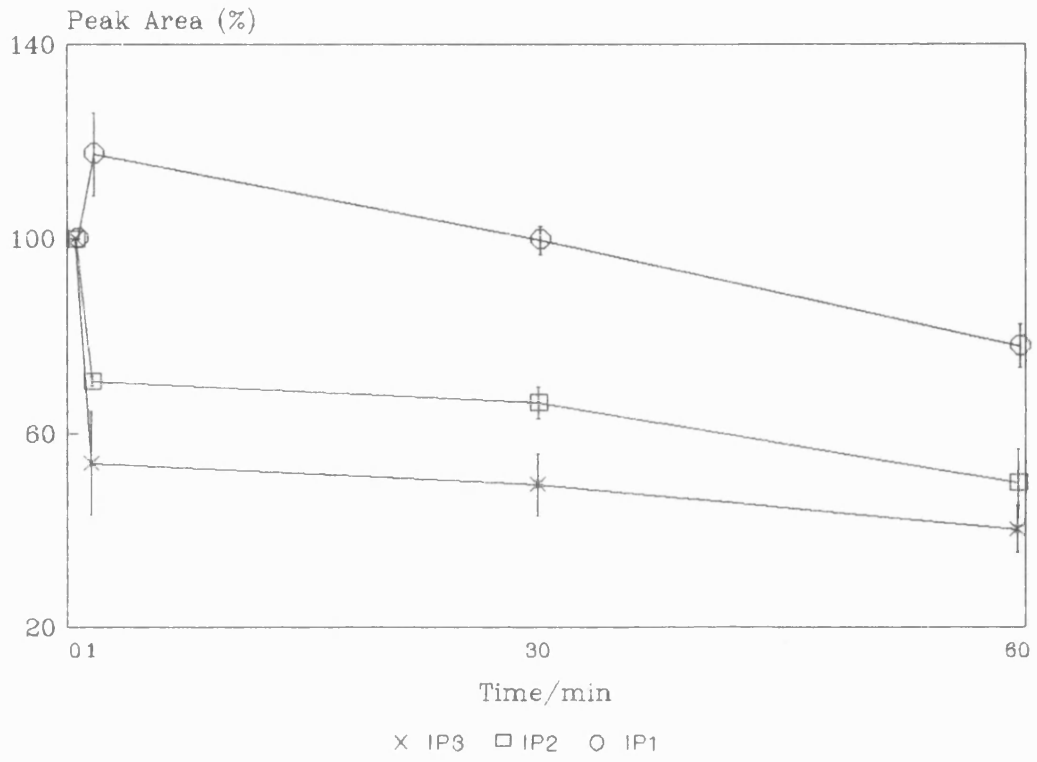


Fig. 3.36. Duplicate Hanson (1991) Style IP_3 Phosphatase Assay

Strain MC3 was grown to the mid-exponential phase of growth and harvested by centrifugation. The cells were washed and broken by Braun homogenisation in 1.5 mM HEPES/0.1 M KCl. The methodology of Hanson (1991) was then followed exactly (described in Materials and Methods 2.18, 2.19). Samples were analysed by elution from a 10 µm SAX anion exchange HPLC column and fractions (2 ml) were collected. These were mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation counting. Area under the curve values were calculated by weight.

trisphosphate peak, the assay was repeated with lithium chloride (12 mM final concentration) in the incubation buffer. The presence of lithium ions in the buffer appeared to have no effect on the degradation of IP_3. The profile showed exactly the same behaviour as the previous assay. A brief increase in the IP_1 levels was accompanied by the general reduction of all three peaks with time. The result implied that the disappearance of the inositol phosphates was not part of a regulated degradation pathway. If the purported phosphatases were behaving as in mammalian cells, lithium would inhibit the conversion of IP_1 to inositol. Maybe the lithium concentration was not high enough to inhibit the enzyme(s) but Delvaux *et al.*, (1987), Ragan *et al.*, (1988), Inhorn and Majerus (1987) and Hughes and Drummond (1987) have all demonstrated marked monophosphatase inhibition at 12.5 mM or lower Li^+ concentrations.

The activity of a Hanson (1991) style enzyme preparation had not been established with respect to the degradation of exogenously added IP_1. An experiment was therefore performed to monitor the breakdown of ^{14}C-IP_1. Using exactly the same methodology, without lithium, area under the curve analysis showed no major decrease in the IP_1 peak during a 60 min incubation (data not shown). The expected result was a reduction in the IP_1 peak to approximately 20% of its original size, as seen for IP_3 breakdown. The observed decrease could be attributed to variations in radioactivity detection levels. It was therefore concluded that the degradation was not derived from a metabolic pathway of

phosphatase activity, involving the progressive phosphorylation and subsequent dephosphorylation of lower inositol phosphates. Although exogenously added ^{3}H-IP_3 was broken down with time, and a transient increase in IP_1 levels recorded, no evidence was obtained for the phosphorylation of IP_2 - the immediate response to IP_3 phosphatase activity. The preparation appeared unable to degrade IP_1 if it was added as the sole substrate, so it was not surprising that lithium ions had no effect on the reaction. It was concluded that the phosphatase enzyme degradation pathway for inositol phosphates was either not present in *Sacch. cerevisiae* or was unable to function. A useful experiment to complete the investigation would have been to monitor the potential degradation of exogenously added radioactive IP_2. The tracer was unavailable at the time, but the assay may have provided further information concerning the nature of the putatively present enzymes. If the reduction of IP_2 had followed a similar pattern to that recorded for IP_3, it could be concluded that non-specific phosphatases were functioning in the cell extract. If however the IP_2 was degraded with a coincident increase in the level of IP_1, followed by the degradation of IP_1 and free inositol production, it could be concluded that a phosphatase pathway was present which was specific for IP_2. This may have helped to explain the inability to detect IP_3 in a yeast cell extract, possibly inferring that IP_2 was the second messenger but the previous degradation experiments did not support the hypothesis. It was not possible to demonstrate an inositol phosphate degradation pathway using

an enzyme preparation derived from the methods of Hanson (1991), so no further experiments of this type were done. Evidence for some kind of IP_3 breakdown had been demonstrated so alternative methodologies were investigated.

3.8.1 Assay of IP_3 Phosphatase Activity using Membrane Preparations Produced with Cationic Micro-Beads

Unable to satisfactorily demonstrate the breakdown of ^3H-IP_3 using relatively crude cell extract preparations, it was proposed that the use of isolated plasma membranes might enhance reaction conditions by eliminating cytosolic material and concentrating the membrane components where the enzymes of interest were potentially located. The distribution of the putatively present IP_3 phosphatase between cytosolic and membrane bound fractions was not known but the removal of any potential inhibitors present in the cytoplasm was hoped to enhance any possible activity. It was considered that if the IP_3 phosphatase enzyme of yeast was predominantly membrane-bound, such a concentration of this cellular fraction would facilitate activity detection. It was also proposed that the use of harsh cell-breaking techniques such as homogenisation may have damaged the enzyme, rendering it unable to degrade IP_3 - the use of charged micro-beads would avoid rough treatment of the cells. Plasma membranes were therefore isolated from sphaeroplasts (see Materials and Methods 2.13) that had been surface-labelled with cationic silica micro-beads (Schmidt *et al.*, 1983; Chaney and Jacobson, 1983; Materials and

Methods 2.14). In the first experiment, membrane preparations in assay buffer were mixed with a solution of ^{3}H-IP_3 and incubated at 25 ^{o}C. Samples were taken immediately after mixing and after 30 min. Both samples gave a baseline profile following the release of free inositol. No peaks were detected and it was originally thought that the result was erroneous due to faulty analysis or human error. The presence of the radioactivity counts in the inositol area of the elution profile suggested very rapid degradation of the inositol phosphates. Perhaps the phosphatase activity was so high that the pathway intermediates were fully dephosphorylated to inositol within seconds of the introduction of ^{3}H-IP_3. This would explain the absence of peaks in the T"0" min (taken immediately after mixing substrate and enzyme source) sample and is the kind of activity level which may be expected when dealing with the deactivation of second messengers. A repeat experiment was therefore performed with one slight modification - a sample of the tracer solution was taken before mixing with the membrane preparation as well as the T"0" min and 30 min sample. The result (Fig. 3.37) reinforced the previous observations and showed that the IP_3 peak vanished within seconds of mixing with the membranes. The IP_2 peak was also greatly reduced at T"0" min and also vanished in 30 min. The IP_1 peak did not appear to be affected immediately, but was also greatly reduced in the T30 min sample. There was however no increase in the levels of IP_2 or IP_1 which again implied that the activity observed was not caused by a specific set of inositol phosphate

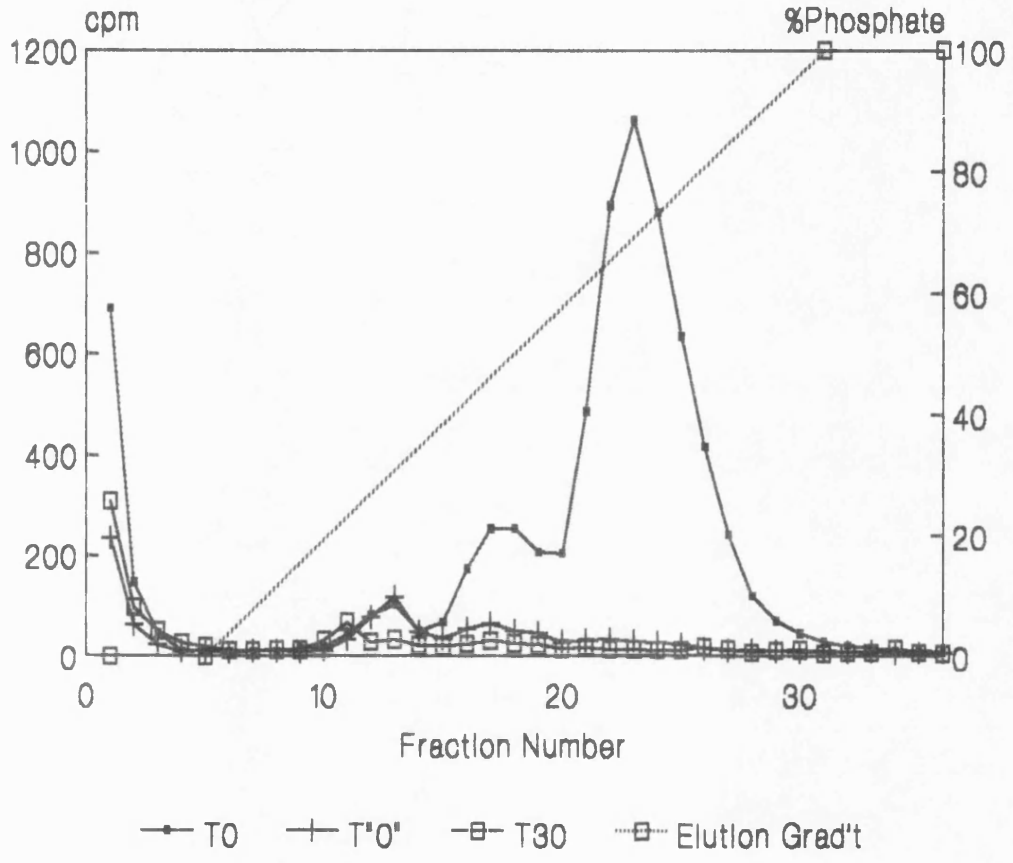


Fig. 3.37. **Repeat IP_3 Phosphatase Assay (Isolated Membranes)** Isolated plasma membranes (Materials and Methods 2.14) were incubated with ^{3}H-IP_3 (0.03 µCi) for 30 min at 25 ^{o}C. Samples (0.5 ml) were removed prior to mixing, then 0 and 30 min after. Reactions were halted by the addition of an equal volume of ice-cold pyridine solution (see text). Samples were eluted from 10 cm Dowex resin columns using a linear gradient (0-100%) of ammonium dihydrogen orthophosphate and fractions (2 ml) were collected and mixed with Optiphase 'Safe' for detection by liquid scintillation counting.

phosphatase enzymes. Exactly the same result was obtained in two repeat experiments. Hence, either the trisphosphate had been fully dephosphorylated back to inositol, which could then be inhibited by lithium, or the radioactivity had been sequestered into an unavailable form and taken into the membranes.

Experiments were done to demonstrate that at least 90% of the original radioactivity could be recovered from ^{3}H-IP_3 samples following treatment with both the TCA and pyridine solution reaction halting procedures (data not shown). It was thus concluded that the massive loss in IP_3 radioactivity was not occurring at this stage. It was also essential to demonstrate that the level of ^{3}H-IP_3 remained constant in the buffer and did not degrade with time. A series of experiments was therefore run to establish the behaviour of potential ^{3}H-IP_3 degradation in a number of buffers, and in the absence of any cellular material. Initially ^{3}H-IP_3 was dissolved in a 1:1 (v/v) solution of distilled water and 30 mM HEPES/$MgCl_2$ buffer. The results (Fig. 3.38) showed a marked decrease in the size of the IP_3 peak with time. The degradation appeared to be non-specific as there was no coincident increase in either the IP_2 or IP_1 peak. It was not known where the radioactivity had gone although the 60 min sample showed an increase in free ^{3}H-inositol content. Further assays were performed with ^{3}H-IP_3 dissolved in (1) distilled water, pH 6.2, (2) 30 mM HEPES/$MgCl_2$, pH 7.0, (3) 30 mM HEPES/$MgCl_2$, pH 7.4 and (4) Membrane Preparation Assay buffer, pH 6.5.

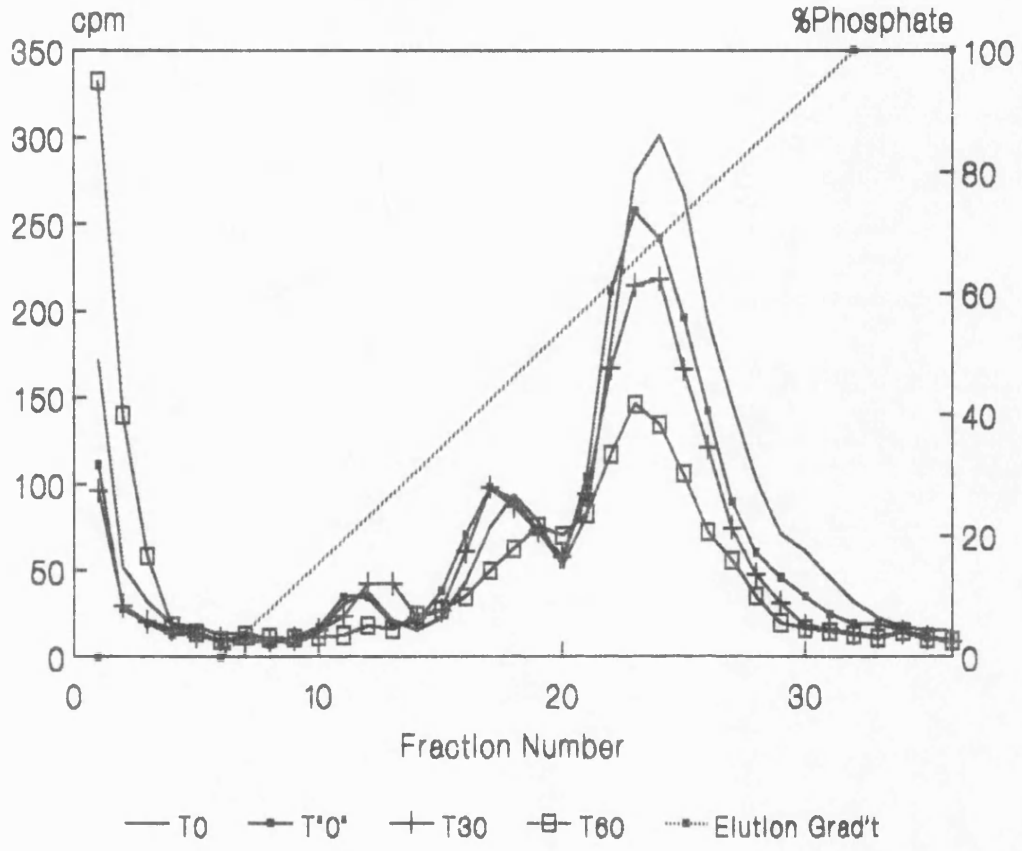


Fig. 3.38. Blank IP_3 Phosphatase Activity Investigation

Tracer ^{3}H-IP_3 (0.03 µCi) was incubated in phosphatase assay buffer (30 mM HEPES/3 mM $MgCl_2$, 4 ml) for 60 min at 25 ^{o}C in the absence of cellular material. Samples (1 ml) were removed periodically and treated with 1 ml of pyridine solution. Samples were eluted from a 10 µm SAX HPLC column using a linear gradient (0-100%) of ammonium dihydrogen orthophosphate, fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation counting.

The methodology used was exactly as described above for each assay and a summary result is shown in Fig. 3.39. It was considered most useful to plot the area under the curve for the IP_3 peak only. When dissolved in buffers 1 and 4, IP_3 gradually degraded with time even in the absence of cellular material. Both of these buffers have a pH below 7.0, and thus the degradation may have been pH dependent. Very little degradation, if any was seen when IP_3 was dissolved in buffers of pH 7.0 or greater (2 and 3), so subsequent assays where always performed at neutral or slightly alkaline pH. Although not illustrated, the data obtained from buffers 1 and 4 showed large increases in the size of the IP_2 peak as the IP_3 peak was reduced. This implied degradation by dephosphorylation of the trisphosphate, and not a general deterioration of the radioactive species. Even though it had been demonstrated that ^3H-IP_3 was degraded by incubation in buffers of pH below 7.0, this breakdown was not fast enough to explain the rapid disappearance of the radioactivity when mixed with membrane preparations. It was possible however to show that neither the buffer nor the reaction-halting step caused the disappearance of the inositol phosphate peaks within the first few seconds of the incubation.

Duplicate experiments performed with the membrane preparations suspended in 30 mM HEPES/$MgCl_2$ pH 7.0 buffer showed exactly the same result as before. The radioactivity was reduced to a baseline only seconds after mixing. Changing the reaction buffer appeared to have no effect whatsoever. It was not known at this point where the

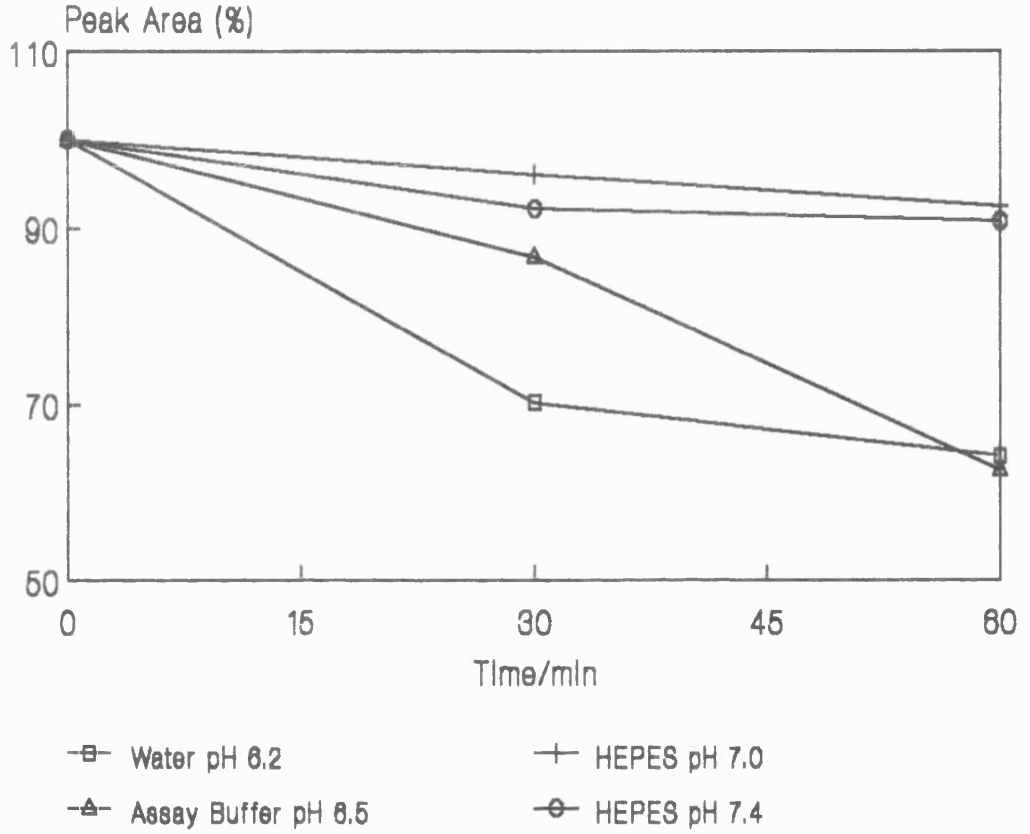


Fig. 3.39. Summary of Blank IP_3 Phosphatase Experiments
Tracer ^3H-IP_3 (0.03 µCi) was diluted in a range of buffers (see text) and incubated for 60 min at 25 ^oC with shaking (120 RPM). Samples (1 ml) were removed periodically and treated with 1 ml of pyridine solution. Samples were eluted from a 10 µm SAX HPLC column using a linear gradient (0-100%) of ammonium formate, fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation counting.

radioactivity was being redistributed to, so to examine further the possibility of sequestration it became routine to perform Bligh and Dyer (1959) extractions on the pellets of the samples. Initial results showed only background radioactivity in both the aqueous and organic layers, but very high counts were recorded in the resuspended pellets. The implication was that the radioactivity was being sequestered either by the charged beads themselves or by some aspect of the membrane preparations that was not affected by the extraction technique.

To examine further the possibiltiy that some enzymatic activity was responsible for the disappearance of the radioactive peaks, it was proposed that evidence could be obtained if the degradation of IP_1 could be inhibited by the presence of lithium ions. It was necessary therefore to demonstrate the degradation of IP_1 using membrane preparations. The result (Fig. 3.40) shows the continuous reduction of the IP_1 peak with time. There was also a coincident rise in the radioactivity in inositol, but it did not reflect the same magnitude of radioactivity in the original IP_1 peak. The degradation of exogenously added IP_1 was demonstrated by membrane preparations but a number of factors suggested that enzymatic activity was not involved. The height of the final inositol 'peak' is less than half of the original IP_1 peak. If the breakdown was purely enzyme-derived, the inositol peak height would have been approximately equal to the T0 min IP_1 peak. Also, IP_1 phosphatase is not normally associated with the membrane

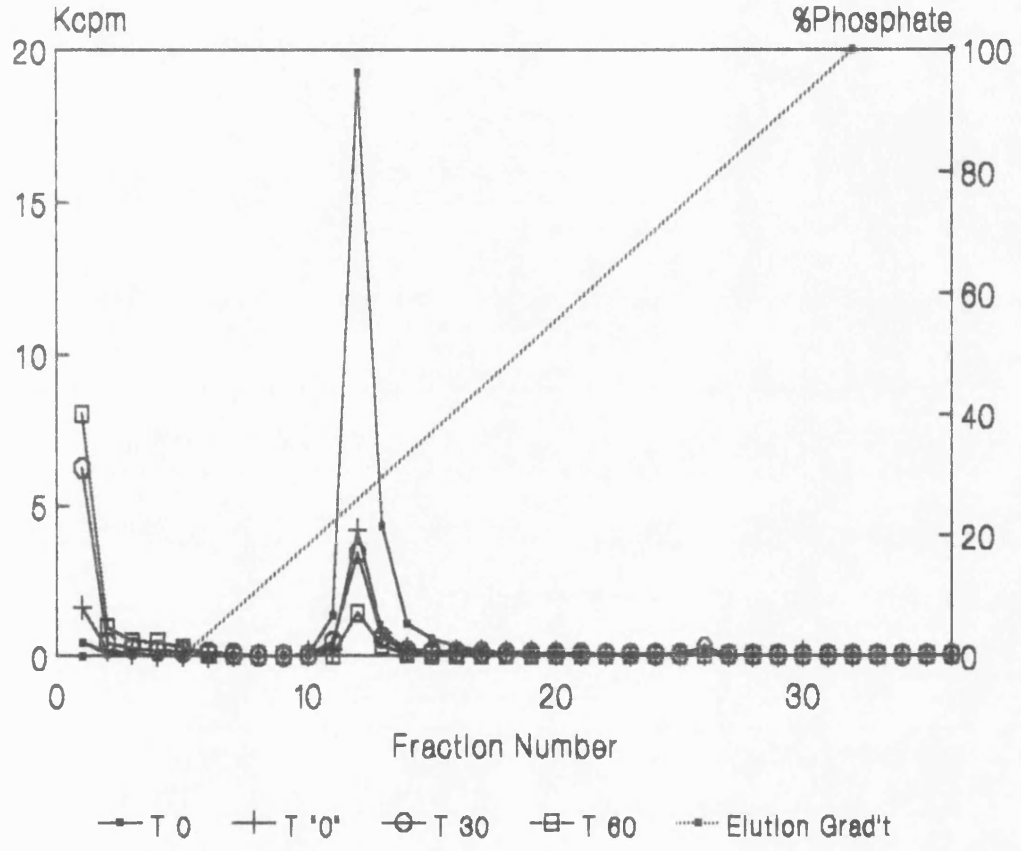


Fig. 3.40. IP_1 Phosphatase Assay Using Isolated Membranes

Isolated plasma membranes (Materials and Methods 2.14) were incubated with ^{14}C-IP_1 (0.03 µCi) for 60 min at 25 ^{o}C. Samples (0.5 ml) were removed prior to mixing, then 0, 30 and 60 min after. Reactions were halted by the addition of an equal volume of ice-cold pyridine solution (see text). Samples were eluted from 10 cm Dowex resin columns using a linear gradient (0-100%) of $NH_4H_2PO_4$ and fractions (2 ml) were collected and mixed with Optiphase 'Safe' for detection by liquid scintillation counting.

fraction of the cell (Downes *et al.*, 1989) and such a high level of phosphatase activity was not expected. Furthermore, it was not possible to inhibit this reaction by lithium ions at a final concentration of 12.5 mM. Lithium ions in an IP_3 phosphatase assay were also completely ineffectual. It was concluded that the observed activity was due to sequestering of radioactivity, so experiments were done to determine if the charged beads or the membranes were responsible.

In and attempt to negate the charge on the cationic beads, the membrane preparations were double washed prior to lysis with coating buffer containing polyacrylic acid (see Materials and Methods 2.14). This had no effect in an IP_3 phosphatase assay but was adopted as routine. It was still not known if the beads were responsible for the sequestering of the radioactivity so to eliminate all possible enzyme activity, a membrane preparation was boiled for 15 min before being used in an IP_3 phosphatase assay. Boiling had no effect on the result and enzyme activity was completely exonerated. In a final experiment to examine the possibility that the radioactivity was taken up by the lipids, a Bligh and Dyer (1959) extraction was perfored on a membrane preparation prior to assay. This again had no effect on the result of an IP_3 phosphatase assay. It was concluded that if sequestering of the radioactivity occurred even in the absence of lipids, the cationic beads must have been responsible. It was not possible to demonstrate any genuine enzyme activity using this methodology - no evidence had been obtained to propose the existence of an inositol

phosphate degradation pathway and so no further experiments of this type were performed.

3.8.2 Investigation of Various Production Stages of Membrane Preparations for the Presence of IP_3 Phosphatase Activity

Although it had been impossible to use cationic bead-derived cell preparations for the assay of potentially present inositol phosphate phosphatase enzymes, the method was investigated further for two reasons. Yeast cells were subjected to a number of different treatments during the production of the membrane preparations and periodically existed in several distinct intermediate conditions. It was not known if any of the intermediate stages, before addition of the charged micro-beads, could be used as a possible enzyme source. Thus, experiments were performed using cells prepared using the same methodology as previously, but the technique was halted at a number of different stages before introduction of the beads. It was then possible to investigate yeast cell preparations for phosphatase activity and define the conditions, if any, in which sequestering of radioactivity occurred.

First, the preparation procedure was halted at the sphaeroplast production stage, and lysed cells were used as the enzyme source. Chromatograms showed the continuous reduction of the IP_3 peak with time (Fig. 3.41). This was coincident with increases in both the IP_2 and IP_1 peaks, suggesting the breakdown of IP_3 was occurring *via* a

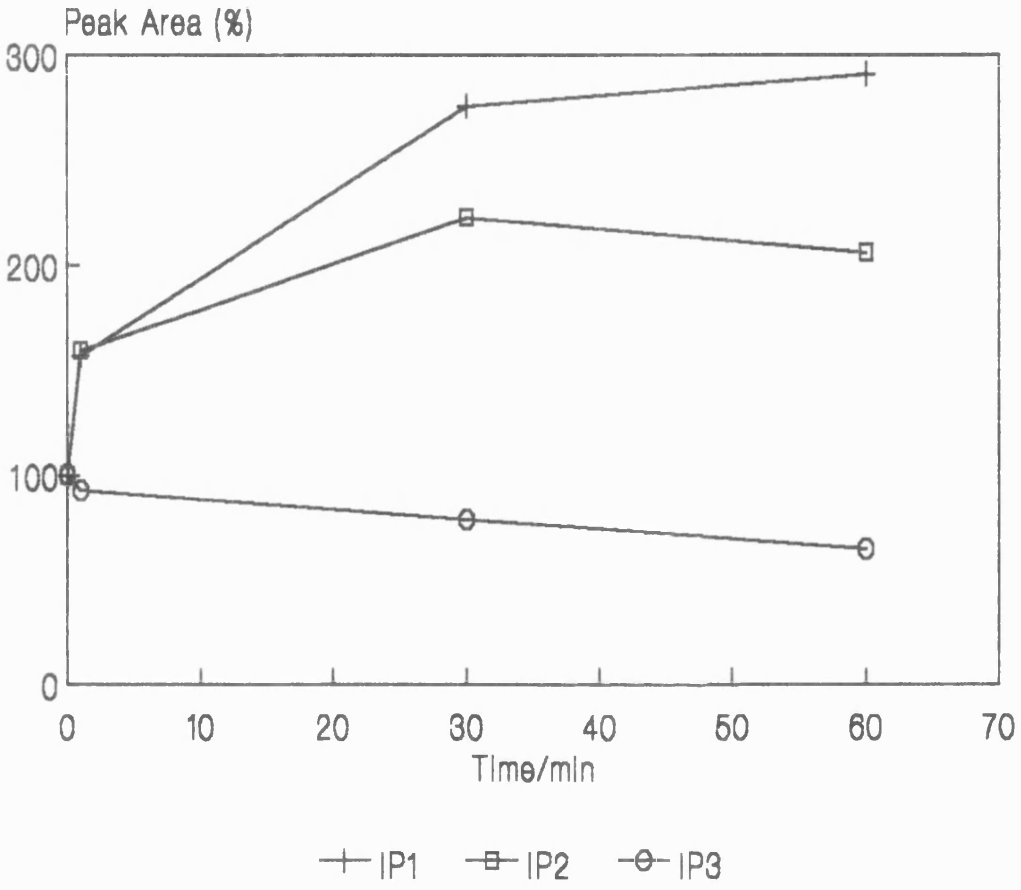


Fig. 3.41. IP_3 Phosphatase Assay Using Sphaeroplasts

The production of isolated membranes was halted at the sphaeroplast stage, and these were lysed by resuspension in phosphatase assay buffer. IP_3 phosphatase activity was assayed as described previously and samples were analysed by elution from 10 cm Dowex anion exchange columns connected to the HPLC. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' for detection by liquid scintillation.

degradation pathway through the lower inositol phosphates. The increase in IP_2 and IP_1 in response to a decrease in IP_3 had not previously been recorded and inferred the existence of a specific IP_3 phosphatase enzyme. The activity was confirmed in a repeat experiment in which an essentially identical result was obtained.

The sphaeroplast supernatant was also assessed for IP_3 phosphatase activity. Following harvest of the sphaeroplasts by centrifugation, a sample of the supernatant (1.5 ml) was taken and used as the enzyme source. An IP_3 phosphatase assay was performed exactly as described above and samples were eluted from 10 cm Dowex anion exchange columns. The result also showed the continuous degradation of the IP_3 peak with time. In this experiment however, there was no concomitant increase in any of the lower phosphates. The result was very reminiscent of the earlier blank IP_3 phosphatase assay (Fig. 3.38) and was not thought to demonstrate genuine enzymatic degradation of the ^3H-IP_3 tracer. During the isolation of plasma membranes, the sphaeroplasts were washed in coating buffer (1.2 M sorbitol, 25 mM sodium acetate, 0.1 M KCl, pH 6.0) and it is believed that the pH of the solution was responsible for the observed degradation of the IP_3 peak. It had already been shown (chapter 3.8.1) that ^3H-IP_3 was unstable in buffers of pH below 7.0 and similar reaction profiles had been observed. The result was confirmed in a repeat experiment but sphaeroplast supernatant was not considered for further investigation of IP_3 phosphatase activity.

The preparation could also be halted at a stage when bead-free membranes were isolated, and these too were used as a potential enzyme source. The bead-free membranes (BFM) were harvested by centrifugation and resuspended in 30 mM HEPES/3 mM $MgCl_2$ buffer (1.5 ml). The phosphatase assay was performed and the result (Fig. 3.42) showed a similar trend to that obtained in the sphaeroplast experiment. A degradation of IP_3 was recorded, coincident with increases in IP_1 and IP_2. The trisphosphate is seen to decrease continuously with time whilst both IP_1 and IP_2 show a rapid increase within the first few seconds of the assay. The IP_2 then begins to reduce slowly in concentration, presumably as it is dephosphorylated to IP_1. The IP_1 curve shows an increase until 30 min, then decreases. A similar result is seen for the sphaeroplast assay, both IP_1 and IP_2 increase rapidly at first, then the reaction rates became considerably slower. A decrease in the level of IP_2 occurs after 30 min in the sphaeroplast result, and although a decrease in IP_1 was not seen, this was probably due to the assay not being run for long enough. Both results provide good evidence for the existence of a phosphatase degradation pathway for the inositol phosphates in yeast. Perhaps the reduction in IP_3 with time is attributed to a randomly acting non-specific phosphatase enzyme; this could also account for the increases in the lower inositol phosphate concentrations. The fact that both IP_2 and IP_1 show a rapid increase followed by a decrease implies more specific activity, suggesting that IP_3 is first converted to IP_2, and that this is then dephosphorylated to IP_1. This is well

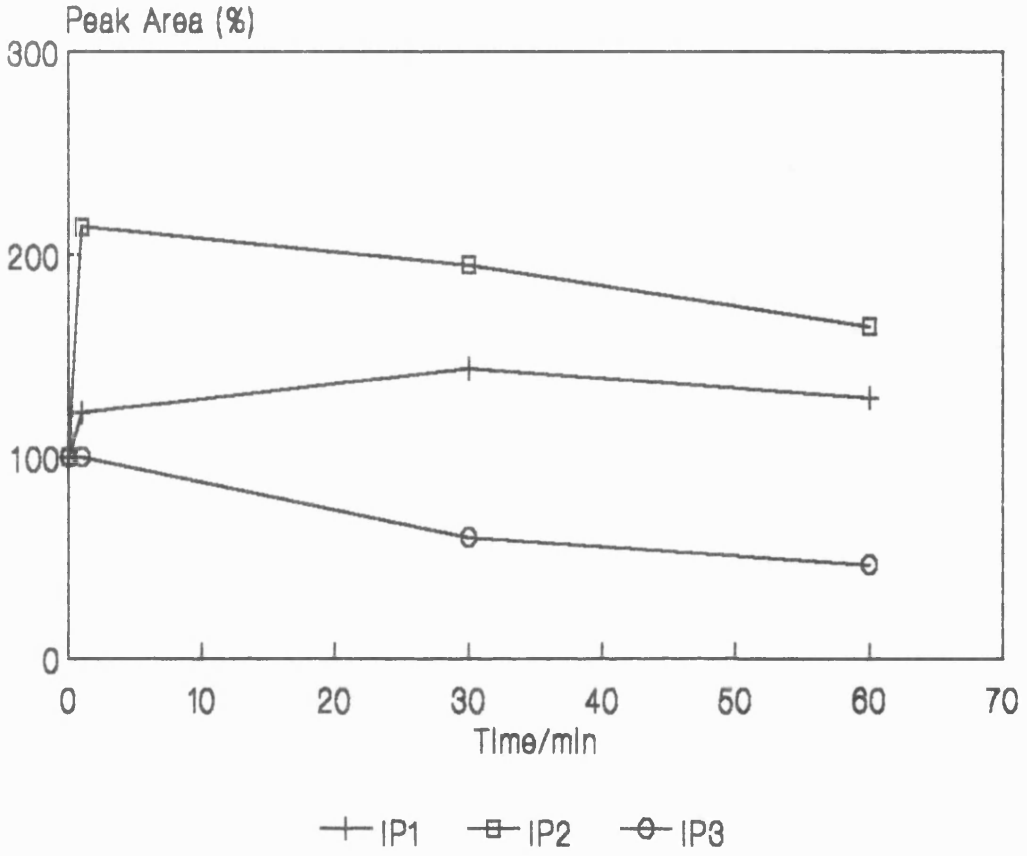


Fig. 3.42. IP_3 Phosphatase Assay Using Bead Free Membranes

The production of isolated membranes was halted at the stage of bead free membranes and these, when resuspended in assay buffer were used as an enzyme source. The phosphatase assay was performed as described (see text) and samples were analysed by elution from 10 cm Dowex anion exchange columns. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation counting.

demonstrated in the result obtained with sphaeroplasts where IP_2 begins to decrease after 30 min but the IP_1 level is still increasing, suggesting that the reduction in IP_2 was contributing to the production of IP_1.

The supernatant from the preparation of the bead-free membranes was also isolated and used as a potential enzyme source. A sample (1.5 ml) was mixed with ^3H-IP_3 (0.04 µCi) in 1.5 ml of 30 mM HEPES/3 mM $MgCl_2$ solution and incubated at 25 ^oC with shaking (120 RPM). The first result was ambiguous and did not indicate the presence or absence of a degradation pathway. A decrease in IP_3 was seen in the first few seconds of the assay, but no further decrease occurred during the next 60 min. No evidence was provided for an IP_3 phosphatase enzyme in this sample. Similarly, both IP_2 and IP_1 showed a small and rapid increase immediately after mixing, but no further increase was recorded until after 30 min. The profile did not resemble those obtained using sphaeroplasts and BFMs and did not show the expected characteristics for a degradation system. A duplicate experiment was performed to confirm the result but different observations were made. The profiles illustrated in Fig. 3.43 were more like those obtained using BFMs and sphaeroplasts. The level of IP_3 decreased throughout the assay and both IP_1 and IP_2 showed continuous increases. It was not possible to explain the very different results obtained using the same preparations but it was thought that the activity seen was derived from solid BFM material remaining in the supernatant. The unrepeatability of the

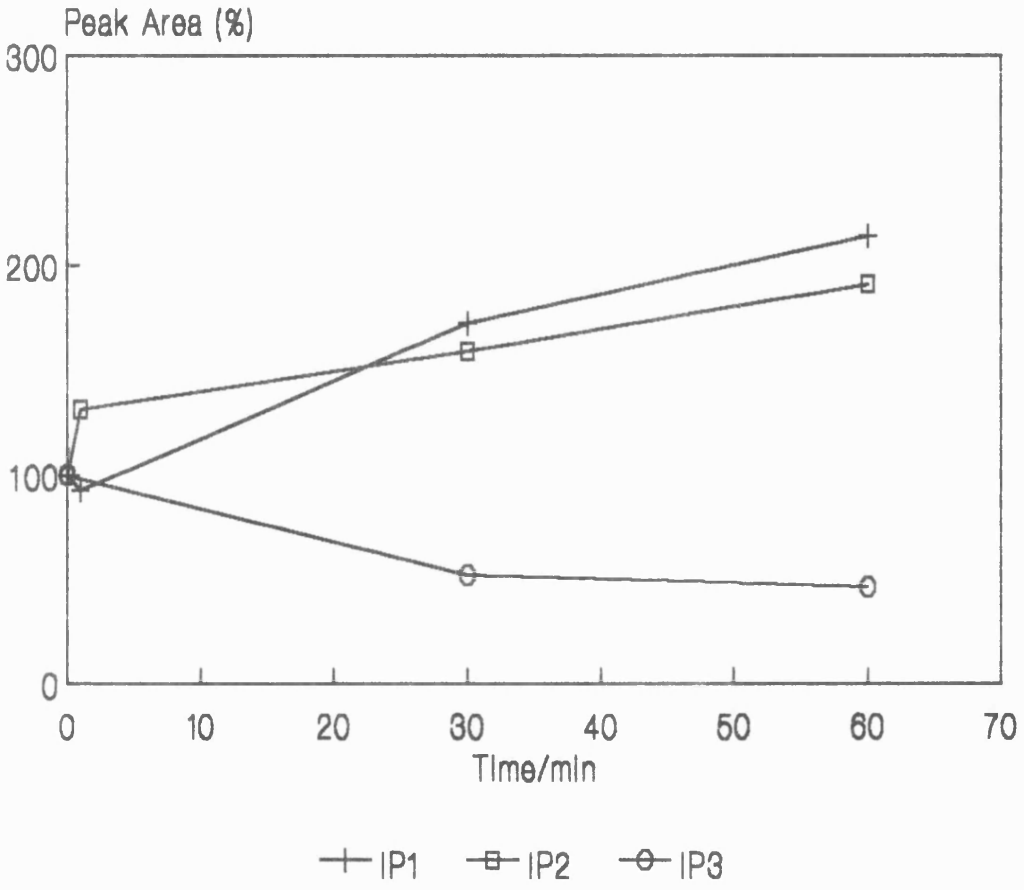


Fig. 3.43. Repeat IP_3 Phosphatase Assay Using BFM Supernatant.

The methodology was exactly as described for Fig. 3.42

assay and the fact that no decrease in IP_2 or IP_1 was recorded made the BFM supernatant an unreliable enzyme source. Even if the phosphatases were present, as suggested in the duplicate experiment, the reactions appeared to be slower than those seen using other preparations. No further experiments were performed using the BFM supernatant as a potential enzyme source.

A final examination was made using the supernatant of sphaeroplasts that had been coated with micro-beads, then lysed as described in Materials and Methods 2.14. An assay of 1.5 ml of the supernatant was performed as usual but there was no overall decrease in the level of IP_3 and no increases were recorded for either IP_2 and IP_1. There was no apparent breakdown of the tracer and the cells appeared to have lost any enzymatic activity they may have possessed by this stage. At no stage was there any indication to suggest that the radioactivity was being sequestered by an enzyme. This gave further support to the theory that the radioactivity was absorbed by the beads, and were unsuitable for the assay of phosphatase activity. Throughout the assays however, evidence had been obtained to indicate the presence of phosphatase activity in yeast cell preparations. It was possible that an inositol phosphate degradation pathway was present in *Sacch. cerevisiae* and to investigate further, a set of experiments was planned to assay potential phosphatase activity and analyse the results using HPLC anion exchange columns. Sphaeroplasts and BFMs were selected as the enzyme sources as these had provided the best

evidence for the system, and HPLC was used to analyse the samples to provide better separation of the peaks and give more accurate and reliable results.

The first of these experiments was performed using sphaeroplasts, and the result (Fig. 3.44) gave further support to the presence of the degradation pathway. Although the rate of reaction appeared quite slow, there was a reduction in the level of IP_3 throughout. This corresponded to increases in IP_2 and IP_1 at 30 min and 60 min. Using this method, it was possible to monitor variations in the level of free inositol in addition to the inositol phosphates. The result showed an increase in the level of inositol during the incubation. This could be described as an expected result because IP_1 is finally dephosphorylated to inositol in other known pathways before re-entering the lipid cycle. The increase in inositol in response to the decrease in IP_3, *via* transient changes in IP_2 and IP_1 is the predicted route for enzymes of the inositol phosphate degradation pathway. The result was confirmed in a repeat experiment in which essentially identical reaction profiles were observed for all the inositol phosphates. The fractions containing the free inositol were collected separately and analysed by gas chromatography (Materials and Methods 2.20). This was to confirm the presence of inositol in the samples and eliminate the possibility that the radioactivity was being distributed to another moiety.

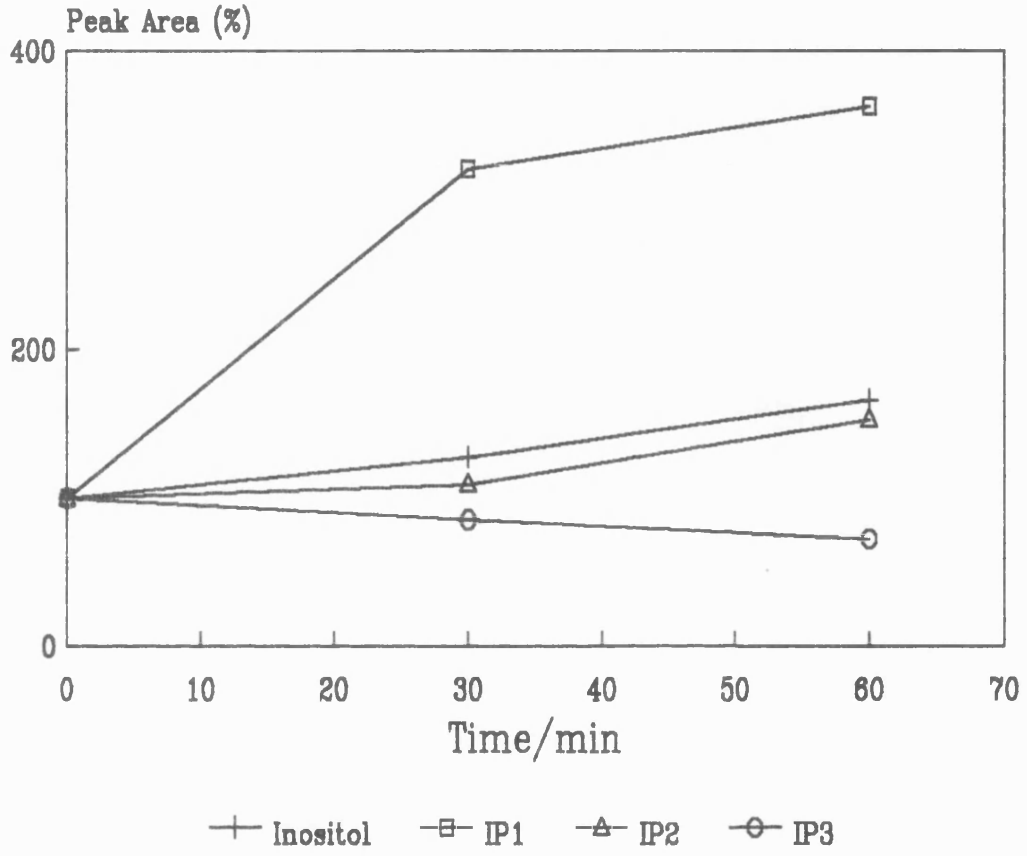


Fig. 3.44. Sphaeroplast IP_3 Phosphatase Assay, HPLC Analysis
The production of isolated membranes was halted at the sphaeroplast stage, and these were lysed by resuspension in phosphatase assay buffer. An assay was performed for IP_3 phosphatase activity as described and samples were analysed by elution from a 10 µm SAX HPLC anion exchange column. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation.

Although only very small peaks were obtained, sample peaks co-eluted with a standard profile and the presence of inositol in the form of ^{3}H-inositol was verified (data not shown).

Similar experiments were performed using bead-free membranes as the enzyme source. The primary result (Fig. 3.45) showed that the inositol phosphate profiles did not follow the patterns seen previously. The level of inositol showed an initial decrease, following which it remained relatively constant. No increase was seen throughout the experiment which was contrary to the expected result. There was a definite decrease in the level of IP_3 but the 30 min IP_3 level was less than at 60 min. This was also unexpected and did not confirm the previous results. The transient appearance of IP_1 at 30 min was evidence for the system but this was not supported by the levels of IP_2, which appeared to fluctuate during the incubation, showing no particular trend. It is possible this was "operator-error" but the experiment was not repeated. Better evidence for the inositol phosphate degradation pathway was obtained using the sphaeroplast stage of the isolated membrane preparation methodology. The sphaeroplasts were not only easier to produce but also had the advantage of retaining both cytosolic and membrane fractions. As the subcellular location of the IP_3 phosphatase was unknown, this was considered to be important. The fact that BFMs had already shown some evidence for the pathway being present indicated some degree of connection to the membrane, but phosphatases

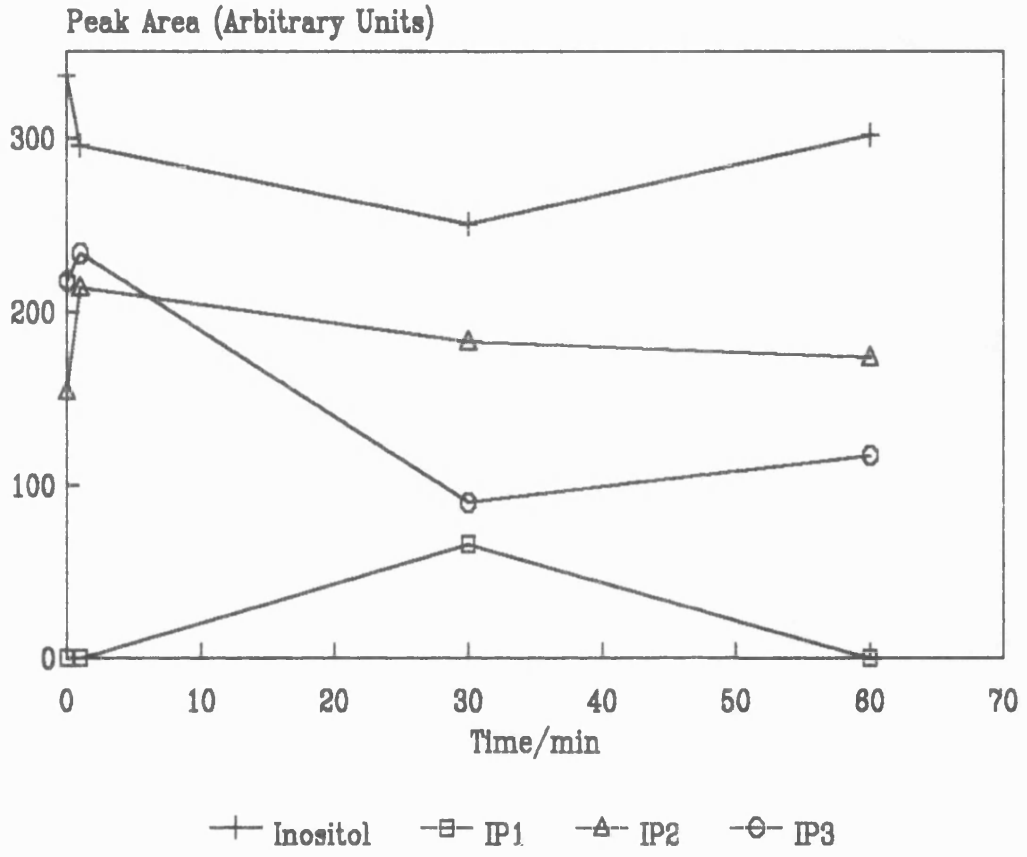


Fig. 3.45. BFM IP_3 Phosphatase Assay, HPLC Analysis

The production of isolated membranes was halted at the stage of bead free membranes and these, when resuspended in assay buffer were used as an enzyme source. The phosphatase assay was performed as described (see text) and samples were analysed by elution from a 10 µm HPLC anion exchange column. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation counting.

for IP_2 and IP_1 were expected to be more prominently cytosolic. Both IP_2 and IP_1 are intermediates that are released solely to the cytoplasm being highly water soluble and hence it was thought that the majority of the enzymes responsible for their breakdown would also be cytosolic. Analysis by elution from the Dowex resin columns showed the sphaeroplast phosphatase assays to give a greater yield of IP_2 and IP_1 and the turnover profiles showed more expected behaviour. Also in favour of the sphaeroplasts was the IP_3 profile in the same result (Fig. 3.41), the reaction rate was still linear at 60 min. The corresponding result for BFMs (Fig. 3.42) showed the IP_3 curve to be levelling out at 60 min and possibly reaching the end of the reaction. It was decided therefore, to perform further experiments using sphaeroplasts only as the enzyme source. As a final investigation into the various aspects of the phosphatase assays using various stages of the isolated membrane methodology, the supernatant from BFMs was examined using a novel technique. Previously, a sample was taken from the neat supernatant and subsequently assayed. In this experiment the supernatant was centrifuged at 40,000 x *g* for 30 min after Hanson (1991). The resulting pellet was then resuspended in 30 mM HEPES/3 mM $MgCl_2$ buffer and used as the enzyme source. The assay then proceded as normal for 2 hours. The result (Fig. 3.46) gave further support for the use of sphaeroplasts as the principal enzyme source. BFM supernatant is composed of the cytosolic fraction of the ruptured cells, and when treated by the method of Hanson (1991), showed that inositol phosphate degradation enzymes

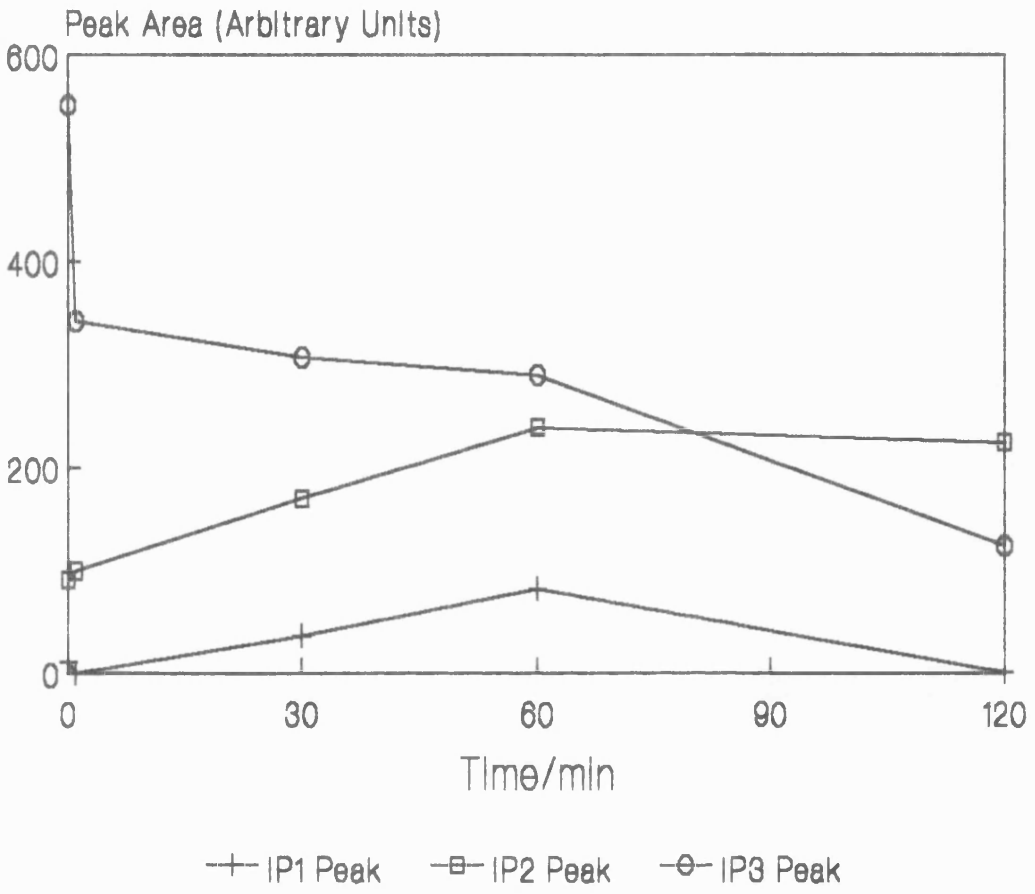


Fig. 3.46. BFM Supernatant IP_3 Phosphatase Assay, New Method
The production of isolated membranes was halted at the bead free membranes stage, and the supernatant was obtained by centrifugation. This was further centrifuged for 30 min at 40,000 x *g* and the resulting pellet was resuspended in phosphatase buffer. An IP_3 phosphatase assay was performed as normal (see text) and samples were analysed by elution from a 10 µm SAX HPLC column. Collected fractions (2 ml) were mixed with scintillant (8 ml) for radioactive counting.

were present. The level of IP_3 showed a continuous decrease, whilst IP_2 and IP_1 showed increases until 60 min, then decreased. IP_2 showed only a slight decrease during the remaining time, but at 90 min the IP_1 peak had vanished completely. It was not possible to show the changes in levels of inositol as fractions had to be collected for analysis by gas chromatography. Using this method, an IP_3 phosphatase assay was performed using sphaeroplast supernatant as the enzyme source. The results were difficult to explain, and did not provide any useful information.

It was decided to perform all further experiments with sphaeroplasts only, and two objectives were established. It was considered important to be able to demonstrate phosphatase activity using different inositol phosphates as the substrates. Better evidence for the existence of the degradation pathway could be given if IP_1 production could be monitored in response to IP_2 degradation, and if IP_1 could be degraded separately. This would help to demonstrate a stepwise nature to the pathway and implicate the existence of different enzymes. It was also intended to run extended IP_3 phosphatase assays. Previous experiments had been run for a maximum of 2 hours and the complete degradation of IP_3 to a baseline level had never been seen. If exogenously added IP_3 could be totally broken down by a cell preparation then it would be possible to gain some data about the specific activity of the enzyme.

To investigate IP_2 phosphatase activity, sphaeroplasts were prepared and mixed with a solution of ^3H-IP_2 (0.10 µCi) in 30 mM HEPES/3 mM $MgCl_2$ buffer. The result (Fig. 3.47) implied strongly that an IP_2 phosphatase enzyme was present. The IP_2 peak showed a continuous decrease in contrast to the level of free inositol that showed a coincident increase with time. An IP_1 peak was present almost immediately after mixing the substrate and enzyme source and remained elevated until 30 min. By 60 min, the IP_1 level showed a decrease. Evidence was provided for both IP_2 and IP_1 phosphatase activity, supporting the observations made in the IP_3 phosphatase assays. The degradation of the IP_2 appeared to have occurred more rapidly than IP_3 had been broken down in previous assays. It was not possible to quantify this because protein concentrations had not been calculated for the sphaeroplasts but a higher rate of reaction was observed for IP_2 phosphatase. This suggested that IP_2 phosphatase may have a higher specific activity for its substrate than IP_3 phosphatase, or that the method is more suited to the isolation of IP_2 phosphatase. If this result accurately represented inositol phosphate phosphatase activity in yeast, it may imply that IP_2 is a more important intermediate than IP_3 in *Sacch. cerevisiae* and the metabolism is geared to removing IP_2 instead of IP_3. This would support the fact that IP_3 remained undetectable from a yeast cell extract and, may implicate IP_2 as a second messenger instead of IP_3.

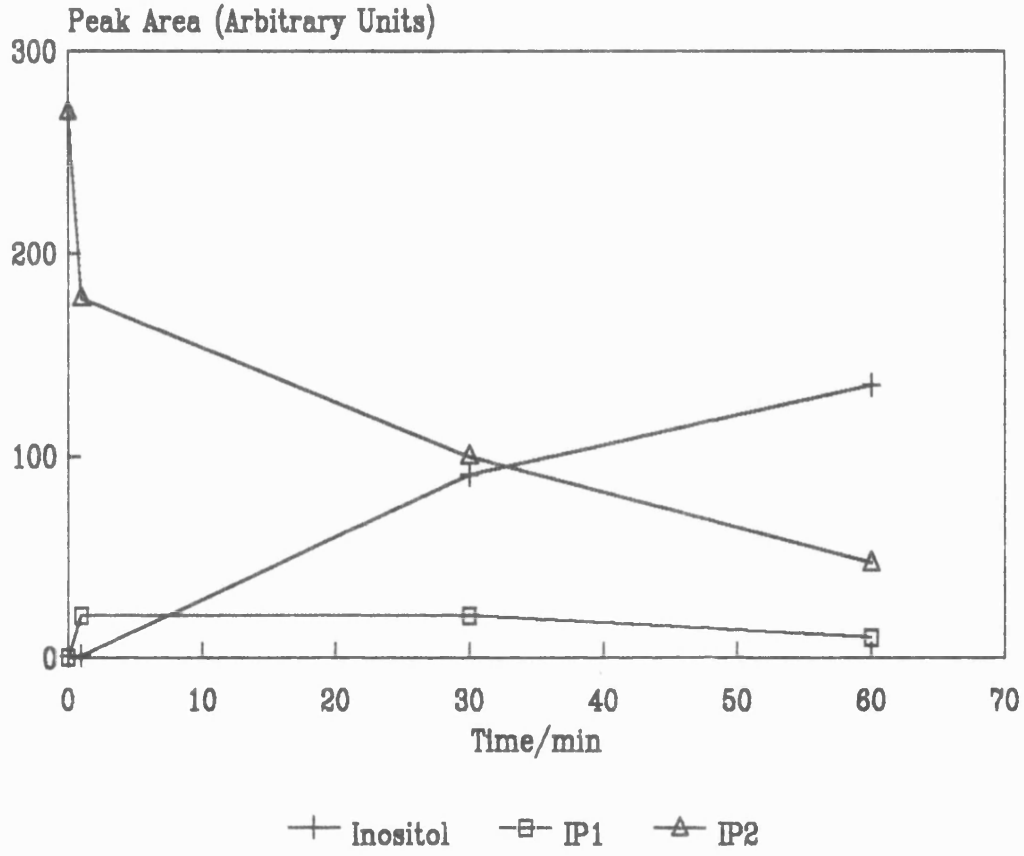


Fig. 3.47. Sphaeroplast IP_2 Phosphatase Assay

The production of isolated membranes was halted at the sphaeroplast stage, and these were lysed by resuspension in phosphatase assay buffer. An assay was performed for IP_2 phosphatase activity (using the IP_3 phosphatase assay methodology) and samples were analysed by elution from a 10 µm SAX HPLC anion exchange column. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation.

Another experiment was performed with ^{14}C-IP_1. The IP_1 peak was degraded with time (Fig. 3.48) with a concomitant increase in the level of inositol (data not shown). This implied that an IP_1 phosphatase was present and fully supported the previous results. The assays had repeatedly shown degradation of the substrate with coincident increases in the levels of lower inositol phosphates and inositol. This was thought to provide an excellent indication for the existence of an inositol phosphate degradation pathway in the yeast *Sacch. cerevisiae*.

Extended phosphatase assays were run with ^{3}H-IP_3 to try and monitor the rate of degradation of the exogenously added trisphosphate. The experiments were performed as previously described but the incubation periods were increased to a maximum of 4 hours. During these investigations, only the IP_3 peak itself was considered, so analysis of the samples was by elution from 10 cm Dowex resin columns. In the first experiment 0.08 µCi of ^{3}H-IP_3 was degraded to less than 20% of its original level during a 4 hour incubation. In the repeat experiment however, an equal amount of tracer was totally broken down in 200 min. The difference in reaction rates was probably due to a difference in protein concentrations, and the very slow nature of the reactions did not imply a very high specific activity for the enzyme, or suggested that the production of sphaeroplasts did not maintain the integrity of the protein. However, since almost total degradation of the IP_3 peak was achieved in 4 h, it was decided to run a similar experiment and analyse the

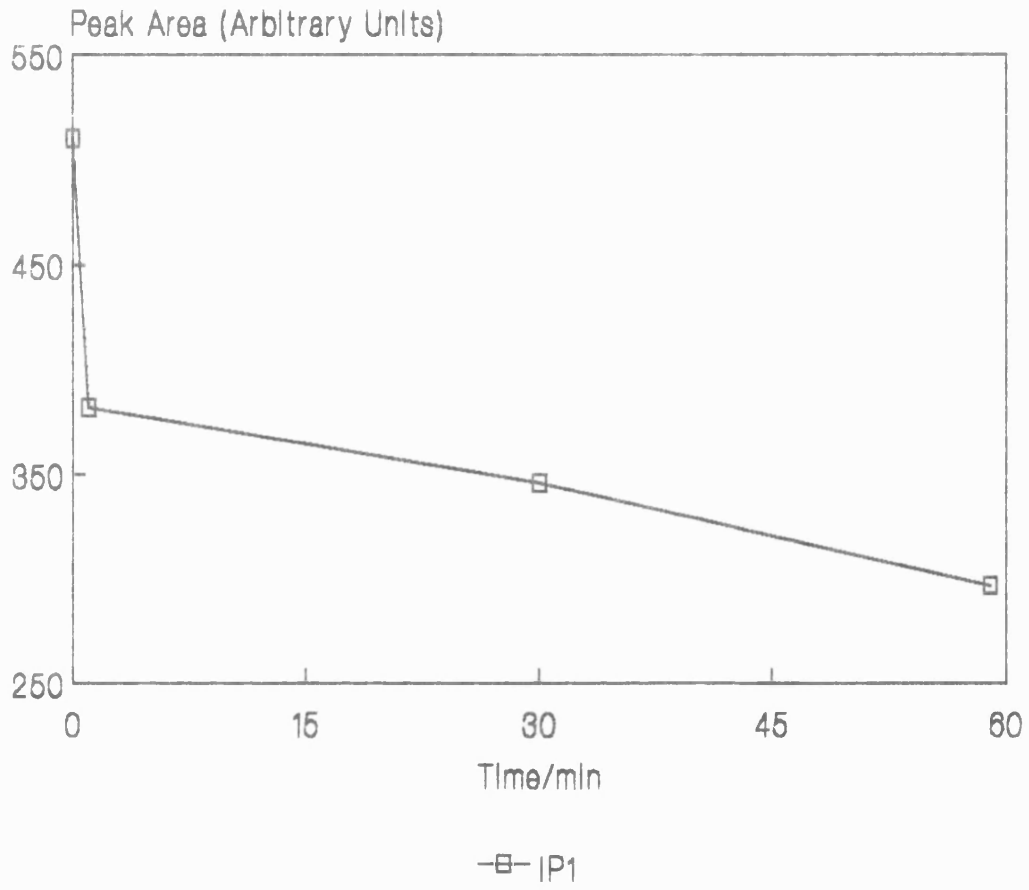


Fig. 3.48. Sphaeroplast IP_1 Phosphatase Assay

Sphaeroplasts were lysed by resuspension in phosphatase assay buffer. IP_1 phosphatase was assayed as per IP_3 phosphatase. ^{14}C-IP_1 (0.1 µCi in 0.3 ml assay buffer) was incubated with a 1.2 ml of the yeast suspension for 1 h at 25 ^{o}C with shaking (120 RPM). Samples (0.5 ml) were analysed by elution from a 10 µm SAX HPLC anion exchange column. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation.

samples using the HPLC. It would then be possible to monitor accurately the turnover of the inositol phosphates and inositol itself. It became standard procedure at this point to wash the sphaeroplasts 3 times in 1.2 M sorbitol (10 ml) before they were resuspended in the phosphatase assay buffer. This was to ensure that all zymolyase had been removed from cell surfaces and would not continue to digest the cells after the designated incubation time. The initial assay was run for 3 hours and demonstrated the expected behaviour of inositol phosphate turnover in the presence of a phosphatase degradation pathway (Fig. 3.49). The IP_3 peak was almost completely degraded during the incubation and reached a baseline level. The level of free inositol increased for the first 2 hours, then appeared to have reached a steady-state which was approximately equal to the IP_3 level at zero time. This was good evidence that the radioactivity had been redistributed between the intermediates of the degradation pathway, and had not been sequestered or released elsewhere. The level of IP_2 showed an immediate increase following the start of the reaction. This can be attributed to the breakdown of IP_3 and continued to increase until the T120 min sample. Following this IP_2 decreased to a baseline level and appeared to be fully metabolised. An increase in the level of IP_1 was not seen until later. If IP_1 production was dependent on IP_2 dephosphorylation, then this was an expected result. As the degradation proceeded IP_2 was produced first, and this was then broken down to IP_1. Without knowing the specific activity of the relevant enzymes, it must be assumed that

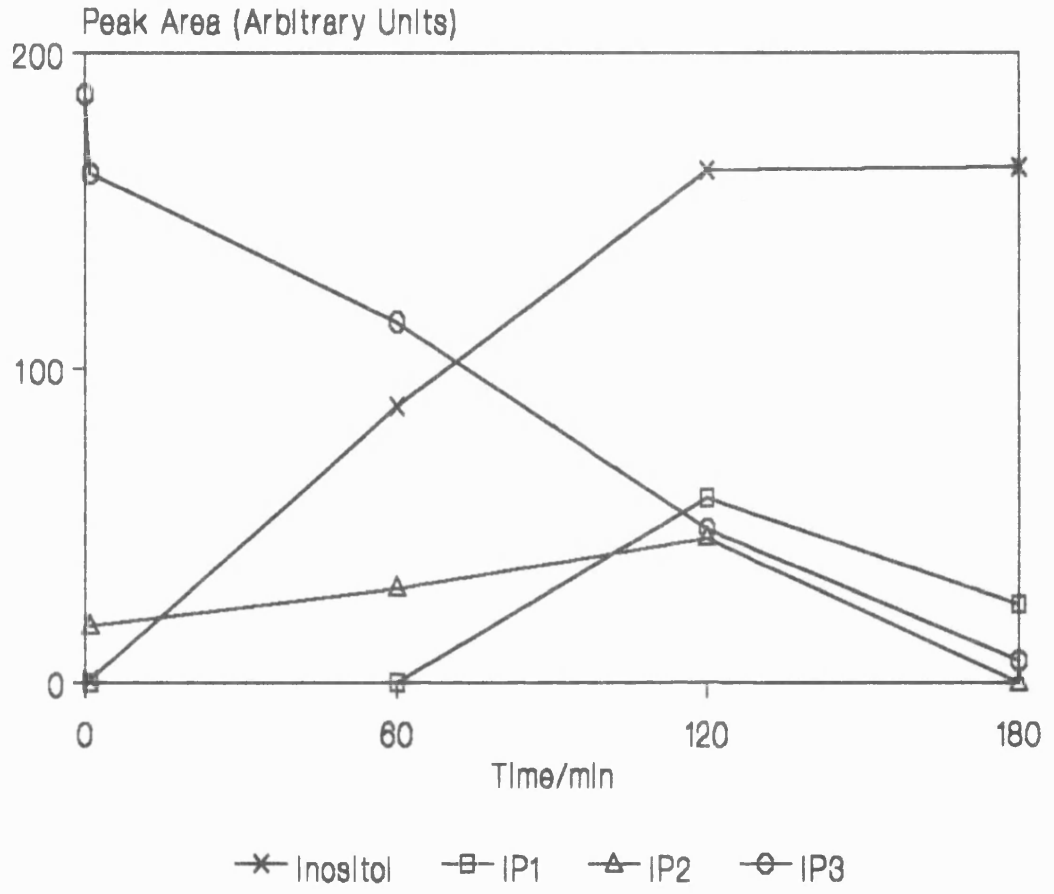


Fig. 3.49. Turnover of the Lower Inositol Phosphates

The production of isolated membranes was halted at the sphaeroplast stage, and these were lysed by resuspension in phosphatase assay buffer. An extended assay was performed for IP_3 phosphatase activity as previously described and samples were analysed by elution from a 10 µm SAX HPLC anion exchange column. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation.

IP_1 can only be produced following the formation and dephosphorylation of IP_2. Overall, the experiment provided good evidence for the degradation pathway. Repeat experiments were performed to confirm the activity and the same trends were obvious in each investigation. However, the reactions could not be inhibited by lithium ions at 12.5 mM (final concentration). This was also noted in earlier experiments using both sphaeroplasts and BFMs in shorter IP_3 phosphatase assays. No inhibition was recorded in any preparation.

A similar experiment was performed with ^3H-IP_2 as substrate. The aim was to monitor the complete degradation of IP_2 using the washed sphaeroplasts as the enzyme source. In previous IP_2 phosphatase assays the breakdown of the IP_2 peak appeared to occur more rapidly than for IP_3. The phosphatase assay was therefore only extended to 2 h. Very good agreement was shown between duplicate assays (Fig. 3.50), but it was not possible to show the complete breakdown of IP_2. In all other respects, the result was another good demonstration of the degradation of inositol phosphates *via* phosphatases. The substrate IP_2 was continuously degraded throughout the incubation which was complemented by an increase in free inositol. However, the inositol concentration did not rise to the level of IP_2 at zero time, but this is probably due to the reactions being halted after only 2 h. It is possible that the curves would have "equilibrated" if the incubation had been allowed to continue. The IP_1 curve showed a small but quite rapid

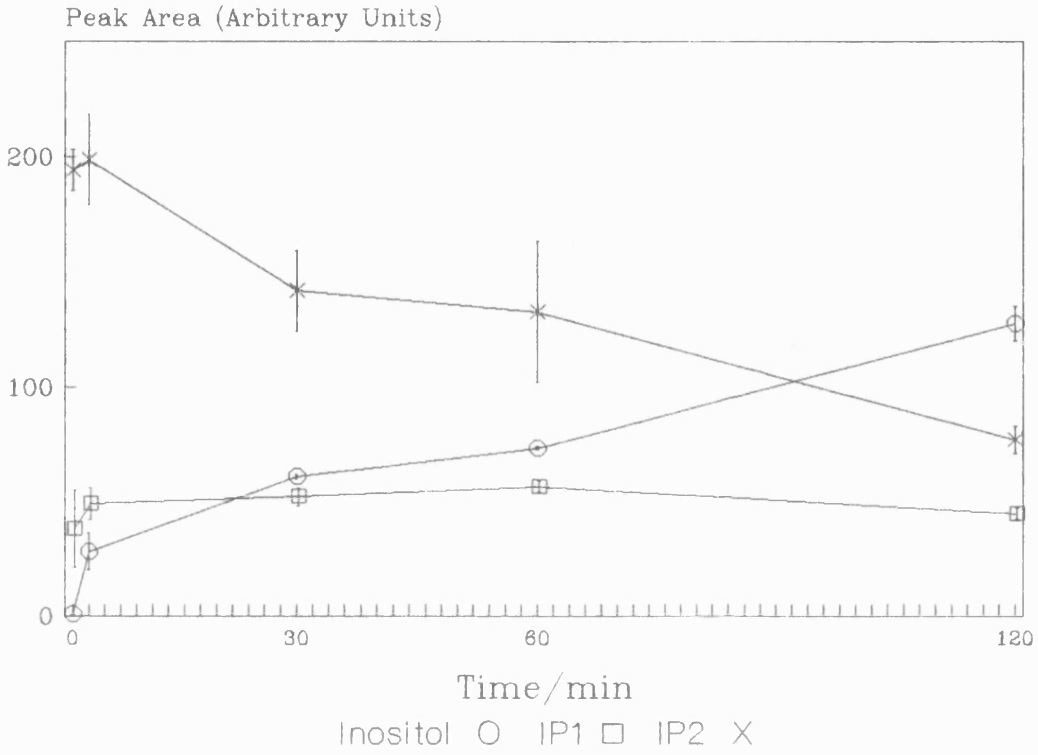


Fig. 3.50. Duplicate Sphaeroplast IP_2 Phosphatase Assay

The production of isolated membranes was halted at the sphaeroplast stage, and these were lysed by resuspension in phosphatase assay buffer. A duplicate assay was performed for IP_2 phosphatase activity (using the IP_3 phosphatase assay methodology) and samples were analysed by elution from a 10 µm SAX HPLC anion exchange column. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation.

increase in the first few seconds of the assay, then slowly increased until 60 min. After this time, a decrease was recorded which would probably have reached baseline level had the reaction continued for long enough. This result fully supported the observations made in the previous experiment using ^{3}H-IP_3 as the substrate. Evidence was provided for a phosphatase degradation pathway for the inositol phosphates in the yeast *Sacch. cerevisiae*.

Although it had now been possible to record the complete degradation of exogenously added ^{3}H-IP_3 using sphaeroplasts, and demonstrate turnover of lower inositol phosphates by way of a phosphatase pathway, it was not possible to assign specific activities to the enzymes involved. The tracer ^{3}H-IP_3 used in these experiments was not fresh and had already developed contaminant IP_2 and IP_1 degradation peaks. This facilitated the detection of variations in the levels of these intermediates but meant that the specific radioactivity of the IP_3 component was no longer valid. Activities could not be attributed to the enzymes because it was not known how much radioactivity was being degraded.

Fresh tracer ^{3}H-IP_3 was therefore purchased and an experiment was performed to examine the distribution of phosphatase activity between the membrane and cytosolic fractions of a preparation of sphaeroplasts. Soluble and insoluble fractions were separated by centrifugation (5 min at 3120 x *g*). The insoluble fraction was resuspended in 1.2 ml of the phosphatase buffer. Both fractions were used as

enzyme sources for use in an IP_3 phosphatase assay to compare the respective activities. The cytosolic fraction contained the highest activity levels. In the solid fraction the IP_3 peak was completely degraded in only 60 min, but peaks corresponding to IP_2 and IP_1 were still present. These were still present at 120 min and the intermediates had not been fully metabolized. In the cytosolic fraction, the lower inositol phosphates had all been fully degraded in 60 min. This indicated much faster turnover in this fraction.

A series of assays were then run in an attempt to calculate the specific activity of the IP_3 phosphatase enzyme in the cytosolic fractions of broken sphaeroplasts. Protein concentrations were calculated for each sample and the rate of degradation of the IP_3 peak was monitored. The results are summarised in Table 3.5, and the mean specific activity of the enzyme was found to be 8.8×10^{-14} mmol mg^{-1} min^{-1} ml^{-1}. Experiment number 2 was not included in the calculations because the sphaeroplasts used in that investigation were found not to have been prepared properly. The cells were prematurely removed from the wall digestion phase of preparation and therefore did not lyse properly. There was very little cell breakage in response to osmotic shock and therefore, the recovery of cytosol was greatly reduced. Even so, the result was not as expected. The rate of turnover was extremely slow and implied that a specific IP_3 phosphatase was not involved. To investigate this further, assays were performed to monitor the rates of degradation of IP_3, IP_2 and IP_1 in a common yeast

Exp^{t} No	Protein conc^{n} mg ml^{-1}	dpm metabolized	Time (min)	Volume (ml)
1	13.13	7 362.9	60	2
2	14.06	2 965.8	120	2
3	13.22	19 865.8	120	2
4	10.52	10 221.8	120	2

Activity in dpm mg^{-1} ml^{-1} min^{-1}

1: 4.67
2: 0.88
3: 6.26
4: 4.05

Mean = 4.99
Std Dev = 1.14

IP_3 Specific Radioactivity : 25.4 x 10^{6} µCi = 1 mM

Specific Activity = 8.8 x 10^{-14} mmol mg^{-1} ml^{-1} min^{-1}

Table 3.5. Determination of the Specific Activity of IP_3 Phosphatase.

The production of isolated membranes was halted at the sphaeroplast stage, and these were lysed by resuspension in phosphatase assay buffer. An assay was performed for IP_3 phosphatase as described previously and samples were analysed by elution from a 10 µm SAX HPLC anion exchange column. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation. Protein estimations were made for each enzyme source and the turnover of dpm was calculated in terms of time, protein content and the concentration of precursor present at the sample times.

preparation. The aim of the experiment was to compare the specific activities of IP_3, IP_2 and IP_1 phosphatases from a single source. Sphaeroplasts were prepared, but soluble and insoluble fractions were not separated. The distribution of enzyme activities for IP_2 and IP_1 phosphatase was not examined, so to ensure maximum recovery of all the enzymes, both fractions were included as the enzyme source. The broken sphaeroplasts were split into three equal samples and an equal volume of tracer IP_3, IP_2 or IP_1 was added. Following analysis by liquid scintillation counting, dpm values were assigned to the peaks and the rates of turnover calculated. The results are summarised in Table 3.6 and show that in these experiments the order of phosphatase activity was $IP_1 > IP_2 > IP_3$. This was a somewhat surprising result and possibly the reverse of what would be expected in a normal inositol phosphate degradation pathway. The very low levels of activity recorded probably indicate that this result does not reflect true phosphatase activities. None of the activities recorded would effectively deactivate a second messenger and turnover at these rates would be almost negligible. This is discussed further in section 4.7. It is notable however that the IP_3 phosphatase activity recorded in this experiment is higher than that in Table 3.5. It may therefore be concluded that the enzyme is distributed between the membrane and cytosolic fractions.

3.9 Investigation of IP_3 Kinase Activity

Evidence had been obtained to imply strongly the existence of an IP_3 phosphatase enzyme in *Sacch. cerevisiae* (Section 3.8.2). The information gathered also suggested that this

Tracer	dpm Turnover	Std Deviation
IP_1	29 985.7	2 146.4
IP_2	28 419.1	5 284.3
IP_3	9 004.0	973.8

All assays were run for 120 min in 1.5 ml of phosphatase buffer and had a protein concentration of 0.64 mg^{-1} ml^{-1}.

Activity in dpm mg^{-1} ml^{-1} min^{-1}

IP_1 = 260.3
IP_2 = 246.7
IP_3 = 78.2

Specific Radioactivity (assuming 100%)

1.22×10^{11} dpm = 1 mM
2.22×10^{12} dpm = 1 mM
5.63×10^{13} dpm = 1 mM

Specific Enzyme Activities mmol mg^{-1} ml^{-1} min^{-1}

$IP_1 = 2.13 \times 10^{-9}$
$IP_2 = 1.11 \times 10^{-10}$
$IP_3 = 1.39 \times 10^{-12}$

Table 3.6. Comparison of Inositol Phosphate Phosphatase Specific Activities

Sphaeroplasts were produced and lysed by resuspension in phosphatase assay buffer. Separate assays were performed for IP_3, IP_2 and IP_1 phosphatase activity and samples were analysed by elution from a 10 µm SAX HPLC anion exchange column. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by scintillation. Protein estimations were made for each enzyme source and the turnover of dpm was calculated in terms of time, protein content and the concentration of precursor present at the sample times.

enzyme had a relatively low specific activity and was in fact the slowest of the three inositol phosphate degrading enzymes investigated. If IP_3 plays the same secondary messenger role in yeast as it does in mammalian cells, it is imperative that the trisphosphate is metabolized quickly and efficiently. In mammalian cells, IP_3 is deactivated in one of two ways:- it can either be dephosphorylated by a phosphatase, or further phosphorylated to IP_4 by a kinase (see Chapter 1). No evidence had been recorded previously for the existence of IP_4 in yeast but experiments were planned to try and demonstrate the conversion of ^3H-IP_3 to ^3H-IP_4 using a yeast cell extract. Not only would this show the presence of an IP_3 kinase in yeast, if the reaction was very fast, it might help to explain the slow activity of the IP_3 phosphatase enzyme. It could then be suggested that, if IP_3 behaves as a second messenger in yeast, it is preferentially deactivated by conversion to IP_4 rather than dephosphorylation to IP_2. The existence of IP_3 kinase would also provide more support for the presence of the phosphatidylinositol turnover systems as a whole, by increasing the number of enzymes known to exist in yeast and providing another possible channel to link the intermediates of the pathway.

To assay the potential kinase activity, broken sphaeroplasts were selected as the enzyme source. These were prepared as previously described and were resuspended in 2 ml of a kinase buffer (50 mM Tris, 5 mM ATP, 5 mM $MgCl_2$, 5 mM Sodium pyrophosphate, 1 mM DTT, pH 8.0) selected from Hansen *et al.*

(1986) and Irvine *et al.* (1986). In the first experiment, it was not clear if a novel peak had been produced. An area of slightly elevated radioactive counts was detectable to the right of the IP_3 peak in the T30 min and T60 min samples but it was not clear if the "peak" was genuine IP_4 or simply a shoulder on the IP_3 peak. A repeat assay confirmed the presence of the "peak" but more strongly implied that it was just a shoulder on the IP_3 peak. The observations were not totally convincing for either argument, so an extended assay was planned. It had been shown in previous phosphatase assays that it was often necessary to incubate for up to 4 h before the full effects of enzyme activity were seen, but in duplicate 3 h assays no evidence of IP_4 production was obtained. It was noted however, that in both assays the IP_3 peak showed evidence of degradation.

An alternative buffer was found for the experiments (Downes, C.P., 1972; pers. comm.) containing 70 mM KCl, 30 mM NaCl, 5 mM ATP, 10 mM $MgCl_2$ and 10 mM HEPES, pH 7.0. Repeat kinase assays were run using the same protocol and a new peak was detected after 30 min incubation in fraction 30 (Fig. 3.51). Although quite small, this was completely separate from the IP_3 peak and had not previously been detected. Apart from the novel peak, degradation of the IP_3 peak was also recorded with coincident increases in the levels of IP_2 and IP_1. This was attributed to the inclusion of $MgCl_2$ in the assay buffer and implied that given the right conditions, exogenously added ^3H-IP_3 can be degraded by pathways which both phosphorylate and dephosphorylate the substrate into

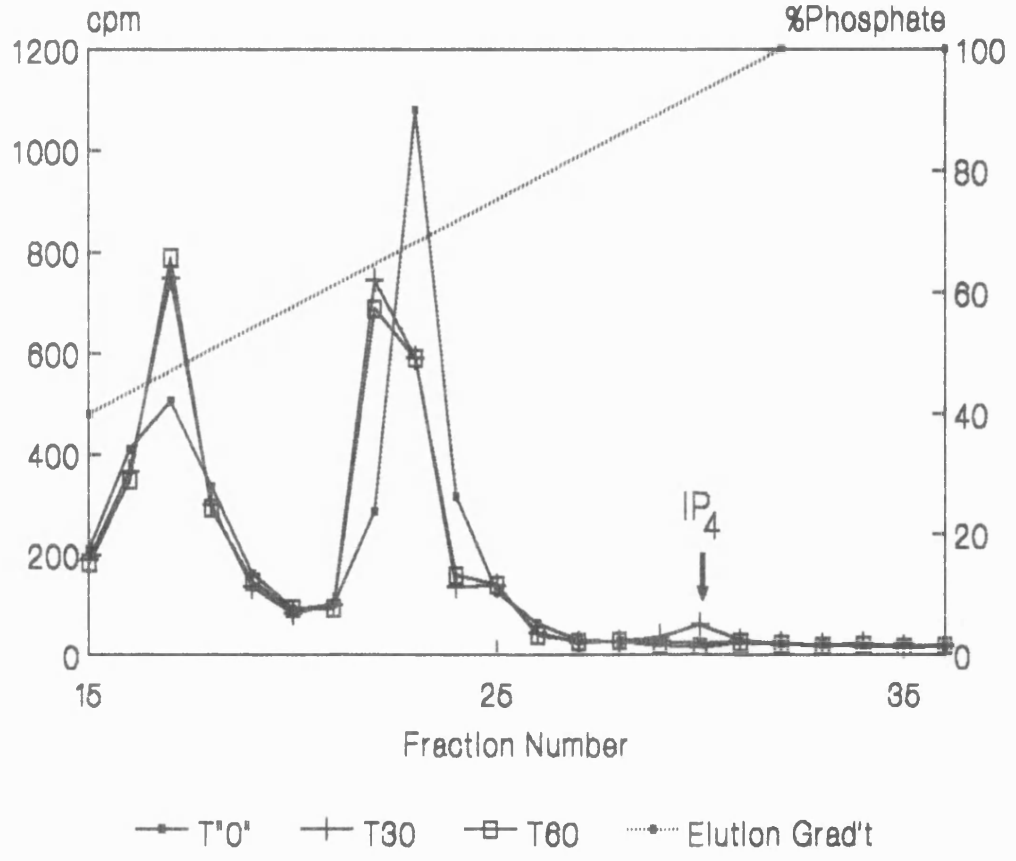


Fig. 3.51. IP_3 Kinase Activity Assay in Improved Buffer
Sphaeroplasts were broken by resuspension in the buffer described by Downes (1992, pers. comm., 2 ml, see text) and the cell preparation was incubated with ^3H-IP_3 (0.1 µCi) for 60 min at 25 ^oC. Samples (0.5 ml) were taken throughout the assay and reactions were halted by the addition of an equal volume of pyridine solution. Samples were analysed by elution from a 10 µm SAX HPLC anion exchange column and fractions (2 ml) were mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation counting.

inactive forms. The novel peak showed the elution profile predicted for IP_4 but its identity could not be confirmed because a standard tracer was not available. In the T60 min sample of the assay, the IP_4 peak had vanished. It is not known how the IP_4 was metabolized but no further peaks were detected. In duplicate experiments, essentially identical results were obtained with an IP_4 peak appearing at T30 min, then showing signs of degradation in the T60 min sample. Also, a decrease of IP_3 was recorded with complementary rises in the levels of both IP_2 and IP_1. It had been shown in repeat experiments that a peak which eluted in the predicted position of IP_4 could be detected if exogenously added ^3H-IP_3 was incubated with a yeast-cell sphaeroplast preparation. This provided good evidence for the presence of an IP_3 kinase enzyme in yeast.

To investigate further the behaviour of the IP_4 peak and to examine the possibility that higher phosphorylated peaks may be produced, an extended assay was run. The result merely supported the observations made in the previous assays. A peak in the position of IP_4 was detected in the T30 min sample which then slowly degraded throughout the rest of the 3 h incubation. No evidence could be obtained for the formation of IP_5 or IP_6 in yeast cells.

Unable to gain any further information from the extended assay, an attempt was made to monitor more closely, the production of the IP_4 peak by running a much shorter assay. The peak had regularly been seen to occur in T30 min samples

of the kinase assays, so a 30 min experiment was run. The result (Fig. 3.52) shows the area under the curve graph of the increase in size of the IP_4 peak. The rate of production however was not particularly fast and the results seen in this and previous assays did not support the theory that the IP_3 kinase enzyme was the major inactivation pathway for IP_3 acting as a second messenger. The observations made in the longer incubations, where IP_3 degradation was directed towards both IP_2 and IP_4 may have indicated some kind of competition between the kinase and phosphatase enzymes of IP_3. Perhaps in this buffer, composed to roughly approximate a yeast cell cytoplasm (Downes, C.P., 1992; pers. comm.) the IP_3 phosphatase had a much higher specific activity, and is able to better utilize the ^3H-IP_3 substrate. This might explain the relatively low levels of IP_4 detected and the fact that it is apparently degraded by dephosphorylation and not further phosphorylated to higher inositol phosphates. It is also arguable that if IP_3 does not play the same second messenger function that it does in mammalian cells, the need for multiple enzymes to inactivate the trisphosphate in two discrete pathways would be greatly reduced, if not redundant. It may well be a reflection on the properties of IP_3 in yeast that the IP_3 kinase activity appears to be very limited.

Tritiated IP_2 was also considered as a possible substrate for kinase activity. As it had not been possible to detect IP_3 in a yeast cell extract in any previous labelling experiment, the detection of IP_2 in such experiments

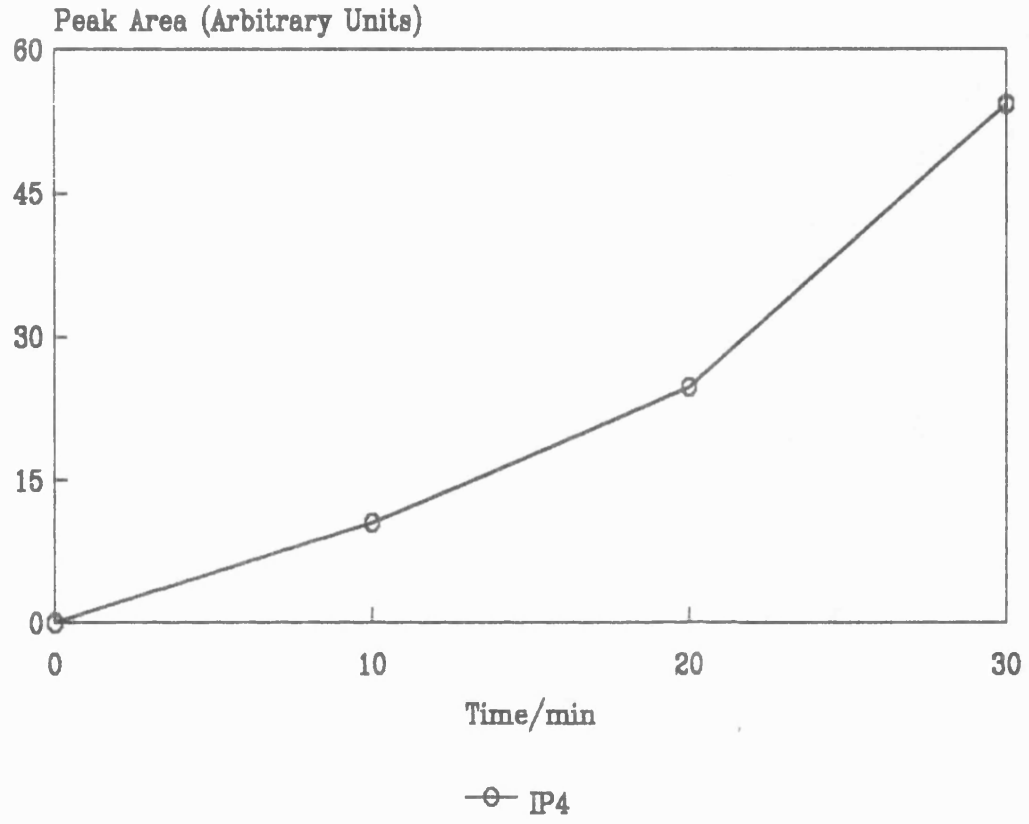


Fig. 3.52. The Production of IP_4 in Broken Sphaeroplasts

Sphaeroplasts were broken by resuspension in the buffer described by Downes (1992, pers. comm., 2 ml, see text) and the cell preparation was incubated with ^{3}H-IP_3 (0.1 µCi) for 30 min at 25 ^{o}C. Samples (0.5 ml) were taken throughout the assay and reactions were halted by the addition of an equal volume of pyridine solution. Samples were analysed by elution from a 10 µm SAX HPLC anion exchange column and fractions (2 ml) were mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation counting.

suggested that the bisphosphate played a more important role. If IP_2 actually played a second messenger role in yeast, then it may be metabolized by more than just the phosphatase pathway already demonstrated. The presence of an IP_2 kinase has not been recorded in mammalian cells and would involve the production of IP_3 without the agonist-mediated hydrolysis of PIP_2. The lack of detection of IP_3 in any yeast cell preparation does not support the existence of an IP_2 kinase, but it is possible that the phosphatase activity over-rode any possible kinase activity, and inhibited the production of IP_3.

No IP_2 kinase activity was detected and the reaction profile was very similar to results obtained in IP_2 phosphatase assays. These observations were duplicated in the repeat experiment where essentially identical results were obtained. No evidence was available to suggest that IP_2 kinase activity existed in yeast. The results indicated that even in a buffer designed for kinase activity very strong phosphatase activity was present, and dominated the reaction profile.

A final experiment of this type was performed to investigate the effect of a long incubation on IP_2 kinase activity. The objective was to discover if an extended IP_2 kinase experiment led to the complete phosphatase degradation of IP_2, or resulted in the delayed production of IP_2-derived IP_3. No evidence of IP_3 production was recorded. The result (Fig. 3.53) shows the complete degradation of IP_2 with time,

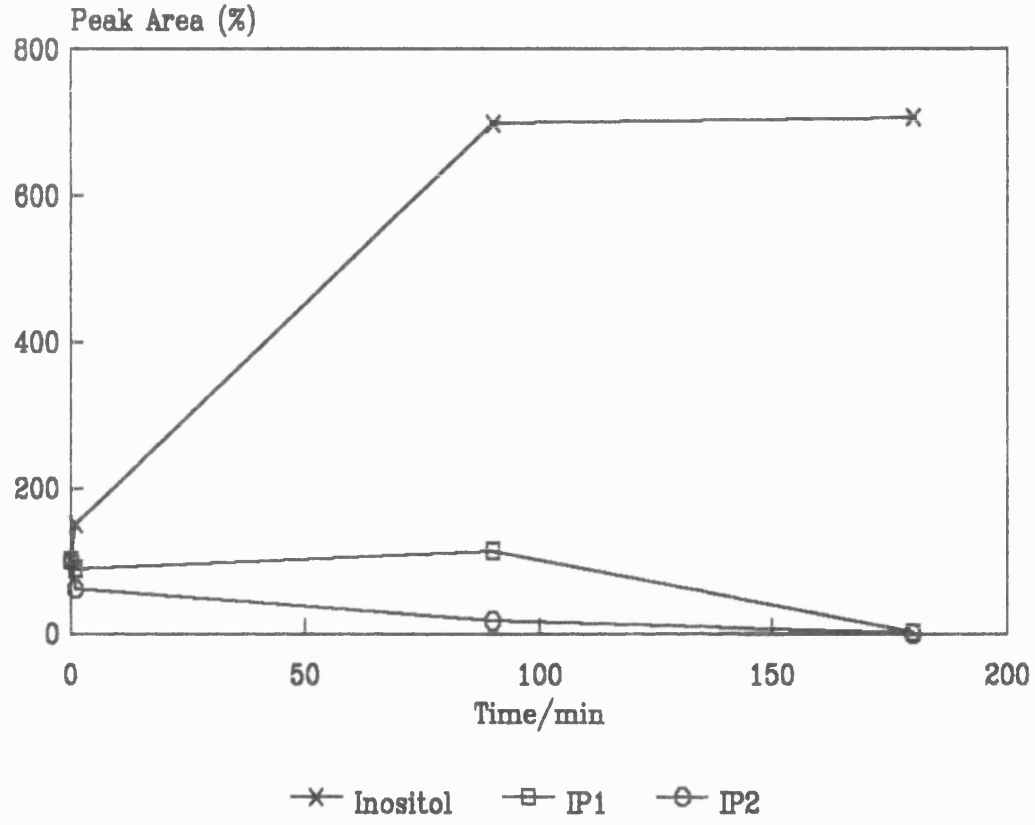


Fig. 3.53. Extended Assay of IP_2 Kinase Activity

Sphaeroplasts were broken by resuspension in the buffer described by Downes (1992, pers. comm., 2 ml, see text) and the cell preparation was incubated with ^{3}H-IP_3 (0.1 µCi) for 180 min at 25 ^{o}C. Samples (0.5 ml) were taken throughout the assay and reactions were halted by the addition of an equal volume of pyridine solution. Samples were analysed by elution from a 10 µm SAX HPLC anion exchange column and fractions (2 ml) were mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation counting.

and a large increase in the level of free inositol. The level of IP_1 shows a small increase until 90 min, and is then completely degraded to a baseline level. It is very unlikely that an IP_2 kinase enzyme exists, which shows similarity with the mammalian system. It also implies that if IP_3 is detected in any further labelling experiments, it must be produced *via* PIP_2 hydrolysis, and not inositol phosphate interconversion. The ability to detect an IP_3 kinase and not an IP_2 kinase suggests that IP_3 is more likely to play a second messenger role in yeast, or at least is a more important metabolite.

3.10 Assay of PIP_2 Phosphodiesterase Activity

The conversion of PIP_2 to IP_3 by receptor mediated hydrolysis is a fundamental step in the PI signal transduction system and the ability to demonstrate this reaction *in vitro* would provide strong evidence for the existence of the pathway in yeast. Experiments were therefore planned to monitor the production of IP_3 when exogenously added tracer ^3H-PIP_2 was incubated with a yeast cell extract. Initial experiments followed the methods described in Materials and Methods 2.10 (method A) and sample columns were cleared of any lower inositol phosphates by thorough washing with an elutant capable of removing IP_2 from the resin. This was an attempt to define better the putative IP_3 peak by ridding the column of all less strongly attached ions. The Dowex resin was then washed with elutant at the concentration to remove IP_3 and it was in these

fractions that a peak was expected. Elution profiles from both the cytosolic and membrane fraction assays showed no peak production in the IP_3 window (Fig. 3.54) and phospholipase C activity was not demonstrated. The peak seen in the "IP_2" window was composed of an unknown moiety possibly derived from PIP_2 itself, which elutes from an ion exchange column. The peak cannot be PIP_2 itself, as the polyphosphoinositide is lipid soluble and should not elute as a single peak in a separation system based on ionic charge in a hydrophilic medium. This PIP_2 "peak" can however be detected if pure tracer PIP_2 is eluted from a Dowex resin column.

Perhaps the incubation period of 10 min was not long enough for turnover to occur, so an experiment was performed for 2 h but similar results were obtained - no IP_3 production in any sample and the expected peak in the IP_2 window. To investigate the possibility that radioactivity was sequestered by the membrane fraction or trapped in an organic matrix, an ether/ethanol extraction was performed to try and recover any PIP_2 or water soluble metabolites. The cells were resuspended in 2 ml of ethanol:diethyl ether (3:1 v/v) and incubated for 10 min at 60 ^{o}C. After 10 min cooling on ice the supernatant was removed after centrifugation (5 min at 3120 x *g*) and the extraction was repeated. The pooled supernatants were eluted from a Dowex anion exchange column, but no further peaks were detected. Perhaps the buffer, being phosphate based hindered any reactions involving the removal or transfer of phosphate groups and so affected the

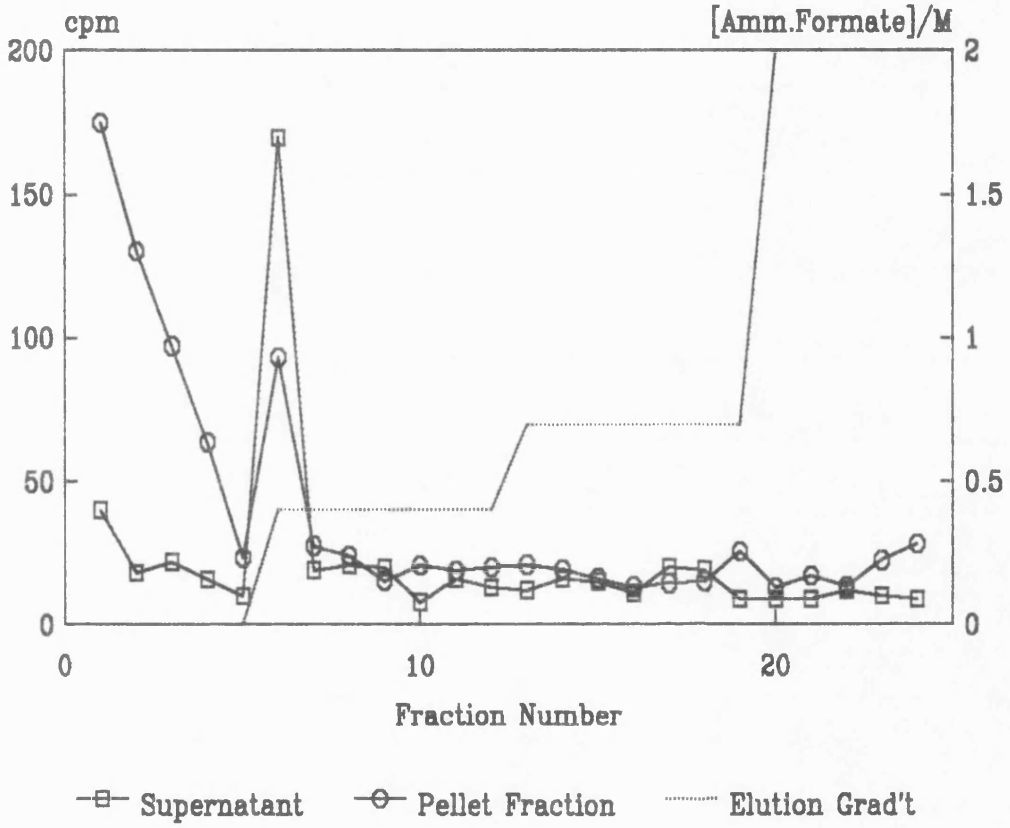


Fig. 3.54. Assay of PIP_2 Turnover in Crude Cell Extracts
The soluble and insoluble fractions of a mid-exponential phase yeast culture that had been broken in a Braun homogeniser were incubated with ^3H-PIP_2 (10 µCi) for 10 min at 30 ^oC. Reactions were halted by incubation at 100 ^oC for 2 min and supernatants were obtained by centrifugation. Samples were analysed by elution from Dowex resin columns (2 ml) using a stepwise gradient of increasing ammonium formate concentration. Fractions (2 ml) were collected and mixed with Optiphase Safe (8 ml) for liquid scintillation counting

turnover reaction. Duplicate experiments were therefore performed using Tris/HCL (pH 7.0) as the reaction buffer. It was still not possible to demonstrate any conversion of PIP_2 and no IP_3 peaks were detected.

Following a review of phosphodiesterase assays in mammalian cells, it was thought that the previous assay may have failed due to lack of essential co-factors. Repeat experiments were planned with an improved buffer after Wilson *et al.* (1984). Using method B, the chromatograms showed no ^3H-IP_3 peaks produced however, and no turnover of ^3H-PIP_2.

Alternative methods for the examination of PIP_2 turnover were investigated, because it is such an important step in the pathway, and it was essential to demonstrate hydrolysis of this phospholipid to give evidence to the theory that a PI based transduction system existed and operated in yeast. As with the IP_3 phosphatase assay, a method to monitor the conversion of PIP_2 became available in the paper by Hanson (1991). The basic methodology was used as described (Materials and Methods 2.18) but the previously prepared 'improved' buffer was retained for the actual incubation. Strain MC3 was grown to a density of 4.4×10^7 cell ml^{-1} in YEPD (400 ml), this was harvested, washed and resuspended in 5-7 ml of a buffer comprising 20 mM Tris/HCL and 1 mM EDTA at pH 7.4. The broken cells were then assayed as described and the result was compared with a sample of the tracer solution removed prior to mixing and treated in exactly the

same way. The result (Fig. 3.55) was not as expected and showed the reduction of the IP_3 peak and the peak in the IP_2 window with time. It had previously been demonstrated that the pyridine solution did not adversely affect IP_3, so the reduction in size was not attributed to this treatment. The result was very reminiscent of the IP_3 phosphatase assays that had been performed using a Hanson (1991) methodology, where the IP_3 peak and lower inositol phosphate peaks had all shown a gradual degradation with time.

Another experiment was run where reactions were halted by the addition of an equal volume of ice-cold TCA (15% w/v) after Downes and Michell (1981). The samples were then left on ice for 10 min and washed with diethyl ether (5 x 2 ml). Following neutralization with sodium carbonate, the samples were analysed by elution from 1 cm Dowex resin columns. An untreated 0 min sample was compared with a treated 0 min sample, and a 20 min sample was collected as usual. The two T0 min samples showed very good correlation but it was noticeable that in the treated T0 min profile, an area of elevated radioactive counts was visible in the final wash window of the elution, corresponding to the 2.0 M ammonium formate elutant which removes IP_4 and higher phosphorylated inositol phosphates from the column. It appeared as if counts had been "dragged" off the column in an unexpected manner. The 20 min sample showed a small peak in the IP_2 window, and a massive peak in the $IP_{4/5/6}$ window. No peak was detected in the IP_3 window and the result was completely different to the experiments that had preceded it.

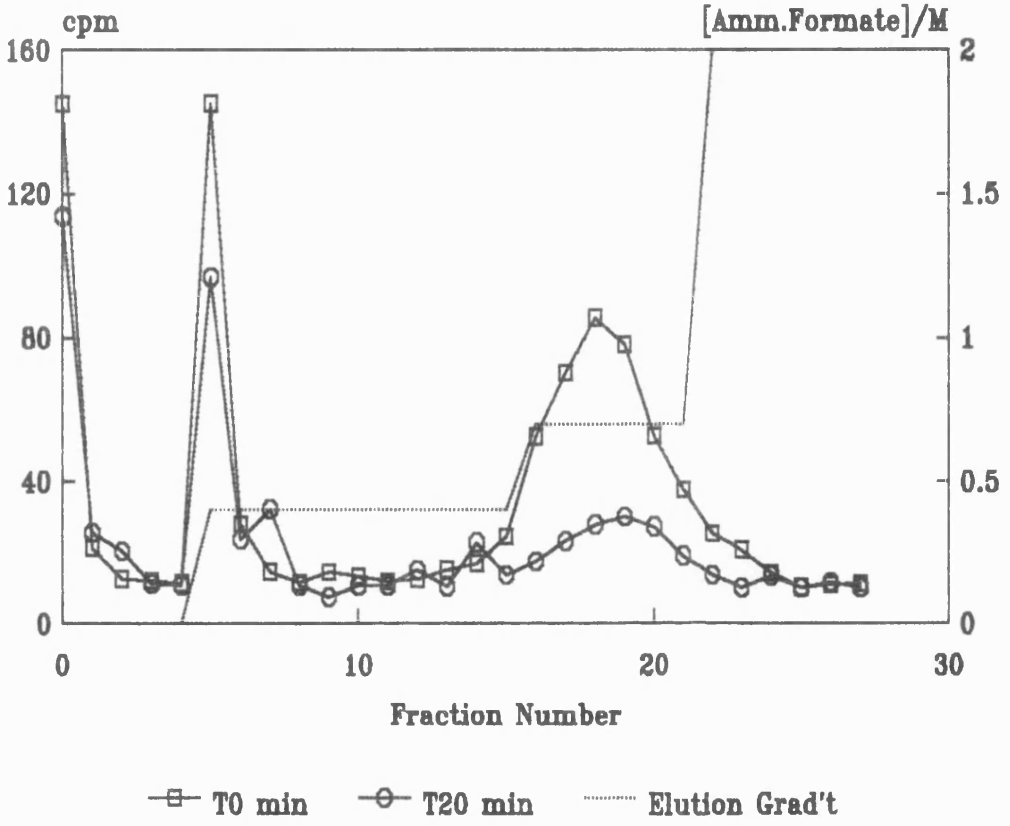


Fig. 3.55. Assay of PIP_2 PDE Activity after Hanson (1991)
A mid-exponential culture of MC3 (400 ml) was broken by Braun homogenisation, and bulk solids were removed from the homogenate by centrifugation (5 min at 3000 x *g*). The supernatant was centrifuged further (30 min at 40,000 x *g*) and the resulting pellet was resuspended in PIP_2 buffer. This was mixed with ^{3}H-PIP_2 (0.05 µCi) and incubated for 20 min at 25 ^{o}C. Reactions were halted by the addition of an equal volume of pyridine solution and analysed as previously described.

In a final attempt to demonstrate ^{3}H-PIP_2 turnover using the Hanson (1991) methodology, a repeat experiment was performed using ice-cold TCA as the reaction halting step and using a 10 µm SAX HPLC column to analyse the samples. The result was different again from those produced on the Dowex resin columns (Fig. 3.56) and each sample showed a peak released in the free inositol position of the chromatogram and a peak in the position of IP_3. Of the peaks in the IP_3 position, there was very little variation and the result indicated that turnover of the precursor ^{3}H-PIP_2 had not occurred. There was some variation in the size of the inositol peaks but the significance of this was not known. The main factor was that there had been no increase in any peaks, or generation of a novel peak during the incubation. The complete lack of evidence for the existence of a phosphodiesterase enzyme and the unrepeatability of the experiments performed meant that further investigations using the Hanson (1991) technique were not carried out.

The advent of the isolated plasma membrane preparations (Materials and Methods 2.14), as used in IP_3 phosphatase assays (chapter 3.8.2) was thought to provide the ideal vehicle for the detection of putative yeast phospholipase C. A concentrated membrane fraction, in the absence of cytosolic components would theoretically isolate the membrane-bound enzyme and facilitate the assay of phosphodiesterase activity. Having eliminated the cytosolic fraction, it was proposed that any IP_3 produced in the turnover assays would not be metabolized as quickly as

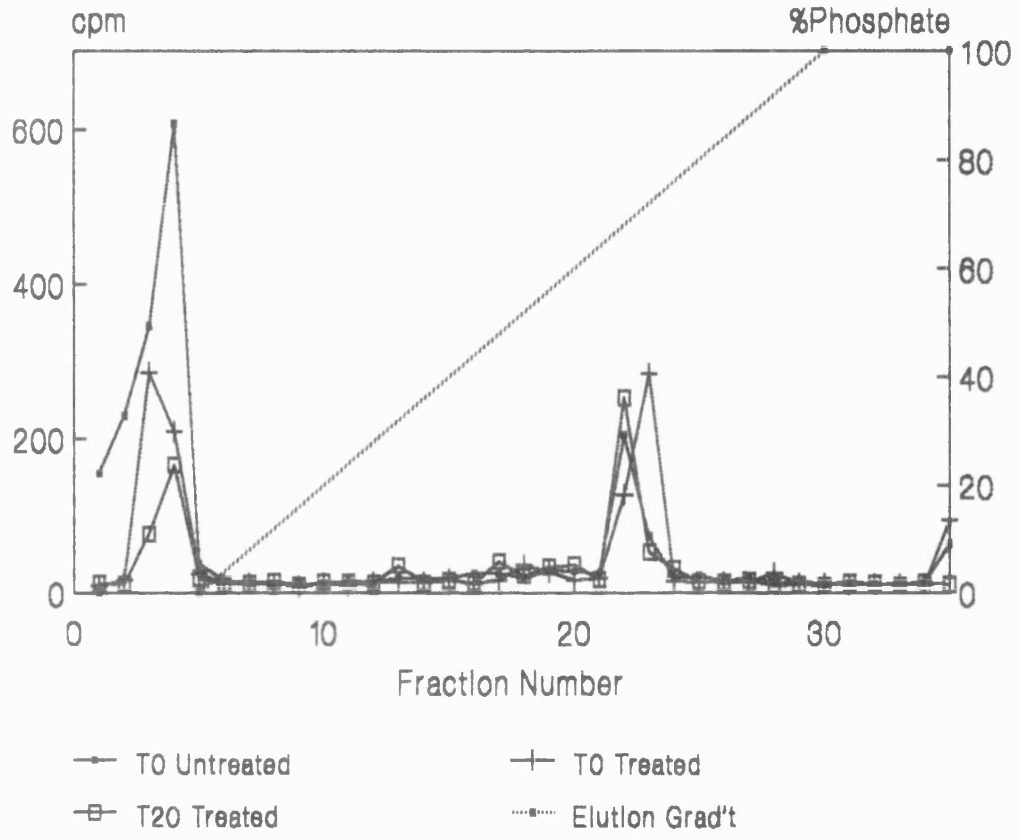


Fig. 3.56. HPLC Analysis of Hanson (1991) PIP_2 PDE Assay
Strain MC3 (400 ml) was grown to mid-exponential phase and cells were prepared according to the method of Hanson (1991). The enzyme source was mixed with ^{3}H-PIP_2 (0.075 µCi) and incubated at 25 ^{o}C for 20 min. Reactions were halted by the addition of ice-cold pyridine solution and supernatants were analysed by elution from a 10 µm SAX HPLC column. Fractions (2 ml) were collected and mixed with Optiphase 'Safe' (8 ml) for detection by liquid scintillation counting.

in previous assays, as water soluble enzymes were not present. Triplicate assays of this type showed no turnover of the ^{3}H-PIP_2 whatsoever. Fig. 3.57 is a typical result showing the presence of a PIP_2-derived peak at 0 min which remained totally unaffected over the 30 min period. No evidence was ever provided for the formation of an IP_3 peak, or for PDE activity. Extending the assay to 2 h had no effect and it was concluded that low activity levels of the enzyme were not responsible for the negative results. Bligh and Dyer (1959) extractions performed on the remaining pellets yielded only background radioactivity, indicating that radioactivity was not being sequestered by the organic components of the preparation, but elevated counts were recorded if the pellet (post-extraction) was resuspended in water.

Although isolated membranes had been prepared in an attempt to enhance any potential PDE activity present, the pellet material was being resuspended in an aqueous buffer. It was therefore proposed that when the hydrophobic ^{3}H-PIP_2 was introduced to the enzyme source, the two components of the assay were not able to interact due to the insolubility of the tracer substrate. The tracer PIP_2 may have formed micelles in the reaction medium and not come into contact with the enzyme source. Repeat experiments were therefore performed, with a detergent in the PIP_2 buffer to aid mixing. Isolated membranes were resuspended in 3 ml of PIP_2 buffer containing TWEEN 80 (0.8% w/v) after Wells *et al.* (1987). The inclusion of the detergent in the buffer

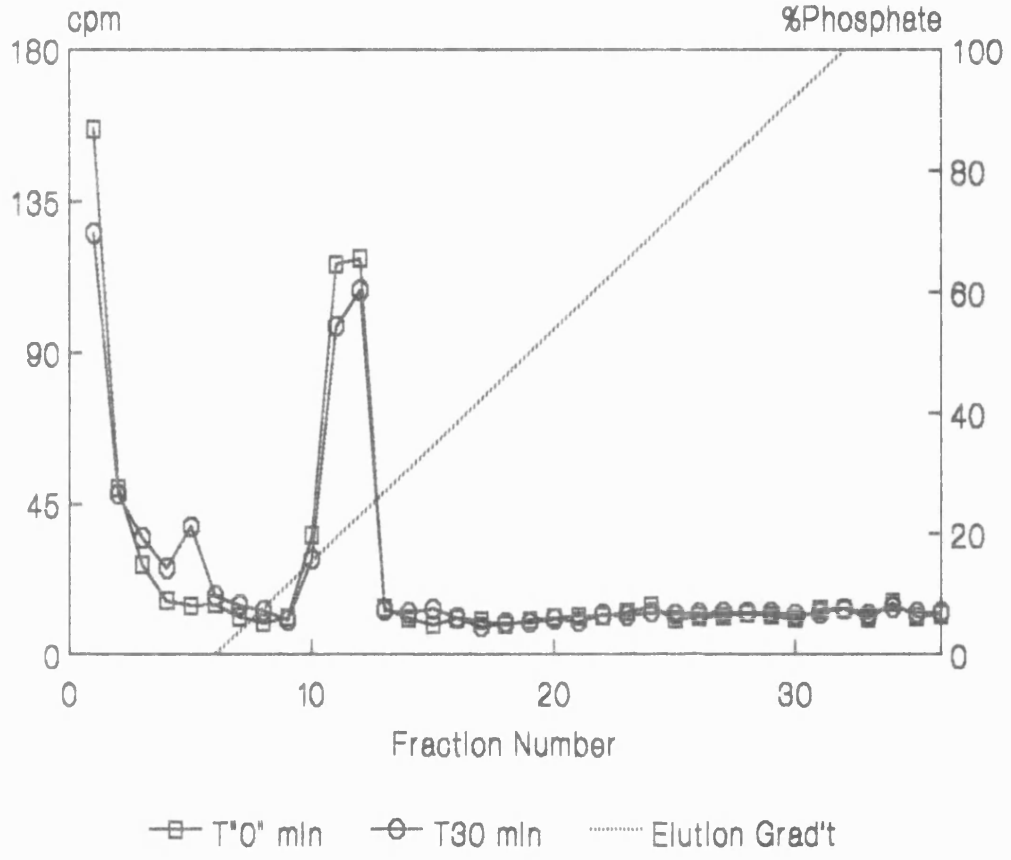


Fig. 3.57. Typical Profile of a PIP_2 PDE Assay Using Isolated Membranes

Isolated membranes were prepared as described in Materials and Methods 2.14 and resuspended in PIP_2 buffer (2 ml). The enzyme source was mixed with ^3H-PIP_2 (0.05 µCi) in PIP_2 buffer (2 ml) and incubated for 30 min at 25 ^oC. Samples (1 ml) were removed and reactions were halted by adding equal volumes of pyridine solution. Supernatants were eluted from a 10 µm SAX HPLC column and fractions (2 ml) were collected and mixed with scintillant (8 ml) for radioactivity counting

appeared to have no effect on the turnover in duplicate assays, no metabolism was detected. Increasing the concentration of the detergent to 1.2% (w/v) also had no effect.

Further experiments with isolated membranes were not performed. The discovery that the silica-microbeads sequestered exogenously added radioactivity rendered them useless. It is notable though that in contrast to the IP_3 phosphatase assays, the ^3H-PIP_2 derived peak remained unaffected throughout. It is not known if this was because the substrate and enzyme source did not mix properly, or due to ionic-charge. Even though no turnover was observed in the ^3H-PIP_2 derived peak, it would have been impossible to monitor the production of ^3H-IP_3. Any trisphosphate produced would have been immediately sequestered by the cationic beads and remained undetectable. No conclusions could be drawn about the existence of PDE activity in yeast from the use of isolated membranes.

Unable to use the isolated membranes as an enzyme source and unsure if inclusion of the detergent in the buffer had had any beneficial effect, PDE assay experiments were performed by returning to the methods of Hanson (1991). The detergent was included so that it would be possible to examine if the previously recorded negative results were due to non-mixing of the enzyme and substrate. No turnover was recorded, and the counts obtained were extremely low. It was thought that the tracer ^3H-PIP_2 may have decayed beyond usefulness, but a

repeat experiment with fresh tracer gave the same result. The presence of the detergent in the assay buffer appeared to have no effect on the attempted detection of PDE activity when using the Hanson (1991) methodology. No evidence has been obtained to suggest breakdown of exogenously added ^{3}H-PIP_2 or the production of IP_3.

To complete this series of experiments a PIP_2 activity assay was performed using a whole cell extract resuspended in PIP_2 buffer containing the detergent. The result appeared to show some kind of turnover activity, although not the expected behaviour. There was still no evidence of IP_3 production but instead showed a novel peak in fraction 9. The observations were not repeatable however and subsequent duplicate experiments to confirm the activity saw a return to the "standard" result where one PIP_2 derived peak was present and remained unaffected.

Prompted by the failure of the isolated membranes to provide a usable enzyme source, bead free membranes (BFMs) were prepared using the same basic methodology. Originally used in IP_3 phosphatase assays (chapter 3.8.2). Cytosolic components were removed, providing an enriched membrane fraction. BFMs were resuspended in PIP_2/TWEEN 80 (1.2% w/w) buffer (1 ml) and an aliquot (1.2 ml) was used as the enzyme source. Assays were performed as described in Materials and Methods 2.19. It was not possible to detect any turnover of PIP_2 to IP_3, but the result (Fig. 3.58) showed the PIP_2-derived peak decreasing with time. The ^{3}H-PIP_2 appeared to

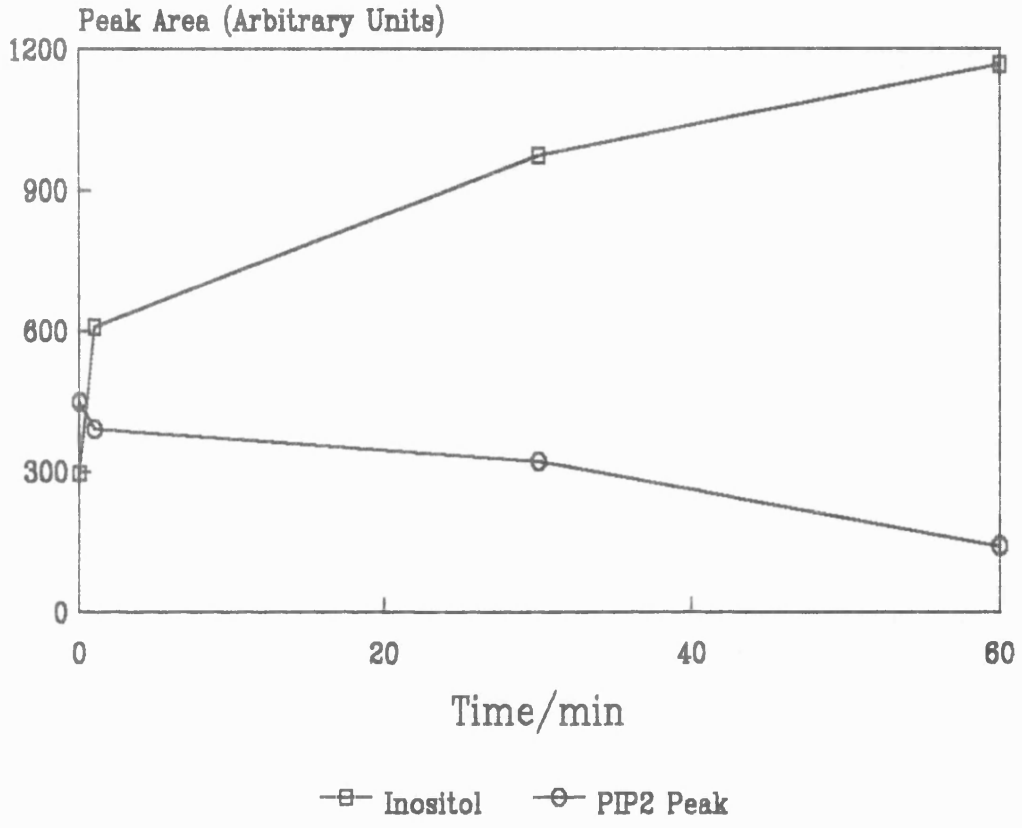


Fig. 3.58. PIP_2 PDE Activity Assayed Using BFMs

Bead free membranes were prepared as previously described (chapter 3.8.2) and resuspended in PIP_2/TWEEN 80 (1.2%) buffer (1 ml). The enzyme source was mixed with ^{3}H-PIP_2 (0.1 µCi) in buffer (300 µl) and incubated at 25 ^{o}C for 60 min and samples (0.5 ml) were removed during the assay. Reactions were halted with equal volumes of pyridine solution and supernatants were eluted from a 10 µm SAX HPLC column. Fractions (2 ml) were collected and counted for radioactivity content in Optiphase 'Safe' (8 ml).

have been affected by some kind of activity, but the only increase in radioactivity in response to the decrease of PIP_2 was in the area of the profile corresponding to free inositol. Using the Dowex resin columns, it was not possible to isolate ^{3}H-inositol but the profiles showed elevated radioactivity in the first few fractions, which increased with time. An extended assay (2 h) to examine the possibility that IP_3 was produced very slowly or only as a result of the complete disappearance of the PIP_2-derived peak showed exactly the same behaviour as the previous one. Perhaps these results represent degradation of ^{3}H-PIP_2 rather than turnover. An IP_3 peak or lower inositol phosphate intermediates were not present to support turnover of the tracer and the reaction rates did not make the instantaneous production of IP_3 and its subsequent degradation to inositol a credible proposition. It is not known if these results indicated a degradation of the ^{3}H-PIP_2 tracer or just the PIP_2-derived peak. The incubation of Li^+ ions (25 mM) appeared to inhibit the degradation of the peak in duplicate experiments. Perhaps the peak was a phosphate-containing part of the PIP_2 tracer which was being slowly degraded with time. The effect of the lithium ions was confusing - the initial objective was to disprove the theory that IP_3 was being produced and degraded extremely quickly. If phosphatases were active in a BFM preparation, it would be possible to inhibit IP_1 phosphatase with lithium. The degradation of the PIP_2-derived peak would continue as normal even in the presence of lithium chloride. Lithium is not known to inhibit the interconversion of the

phosphoinositides and the effect of its inclusion is not understood. The peak was shown not to be a genuine inositol phosphate - a test elution with *bona fide* ^{3}H-IP_2 and ^{14}C-IP_1 added as markers indicated that no co-elution took place. The result could not therefore be attributed to Li^{+}-inhibitable phosphatase activity of the lower inositol phosphates.

It was not possible to demonstrate the conversion of exogenously added ^{3}H-PIP_2 to ^{3}H-IP_3 using a variety of broken cell preparations and conditions. Maybe the lack of activity was due to the inability of the enzyme and the substrate tracer to fully interact because of very different solubilities. Efforts were therefore concentrated on developing a method to incorporate ^{3}H-PIP_2 into the membranes of intact cells. Having the radioactive tracer *in situ* might facilitate the detection of any turnover and avoid potentially damaging preparation steps. Initially, experiments were performed to discover if ^{3}H-PIP_2 was taken up into the cells when present in the growth medium. Strain MC3 was grown in YEPD (100 ml) with 10 µl (0.1 µCi) of ^{3}H-PIP_2 at 25 ^{o}C with shaking (120 RPM) until the mid-exponential phase of growth. There was no reduction in the radioactivity in the medium, which implied that no uptake had occurred. It was possible that the ^{3}H-PIP_2 was taken up and metabolized. The washed pellet was subjected to a Bligh and Dyer (1959) extraction and the radioactive content of the aqueous and organic layers was examined. The aqueous phase was eluted from a 10 µm SAX HPLC column, but produced

only a baseline trace. There was no evidence for radioactivity incorporation in this part of the extract. The organic phase was also found to contain only background counts. A second experiment was performed in which the cells were grown to stationary phase, but exactly the same results were seen. Cell free extract (25 ml) was eluted from a 10 cm Dowex resin column connected to the HPLC. The chromatogram obtained showed the standard ^{3}H-PIP_2 profile and indicated that the tracer had not been metabolized or affected in any way. It was concluded that ^{3}H-PIP_2 was not taken up by yeast cells and it was impossible to incorporate the radiolabel into cell membranes by growing them in the presence of the tracer.

Electroporation was selected as a potential method for incorporating ^{3}H-PIP_2 into the yeast cell membranes. It was hoped that by treating the cells with electrical pulses in the presence of ^{3}H-PIP_2, the tracer would be taken into the cell membrane and subsequently turned over by PDE activity. Following incubation, it was intended to harvest the cells and extract water soluble metabolites, to detect for IP_3 and possibly other lower inositol phosphates.

First, strain MC3 was grown to mid-exponential phase in YEPD. Samples were taken into sterile electroporation vials (Bio-Rad) and 2.5 µl (0.025 µCi) of ^{3}H-PIP_2 were added to each vial. A single pulse of 1.25 kV (1 µFD) was applied to each vial and these were then incubated for 0-60 min at 25 ^{o}C with shaking (120 RPM). The result was difficult to

evaluate since the radioactivity levels were very low and a number of irregular peaks were present that did not correspond to any known inositol phosphate. However in the sample taken immediately after electroporation, peaks corresponding to the ^{3}H-PIP_2-derived peak and the position of IP_3 retention were detected. These peaks were not present in any of the later samples, but it was not known if this was due to metabolism of the compounds or whether the peaks were just counting errors (both had a peak height of <50 cpm). Unable to draw any definite conclusions from this result, a second experiment was performed. The yeast cells were prepared as previously described and in an attempt to improve the level of radioactivity incorporation, a double pulse of 1.5 kV at 1 μFD was applied. The chromatogram (Fig. 3.59) showed what was essentially an improved version of the initial result. Two peaks were again present in the 0 min sample which were not present in any others, but they did not co-elute with the peaks seen previously. It had not been possible to repeat the observations of the first experiment and the non-occurrence of any peaks in the latter samples was not understood. The implication was that degradation of the tracer was occurring, rather than active turnover. The PIP_2-derived peak only occurred in the T0 min samples and its disappearance was not accompanied by a regularly occurring or increasing peak elsewhere.

An electroporation survival experiment was performed where yeast cells (MC3) were grown to mid-exponential phase, and samples (1 ml) were treated with single pulses of increasing

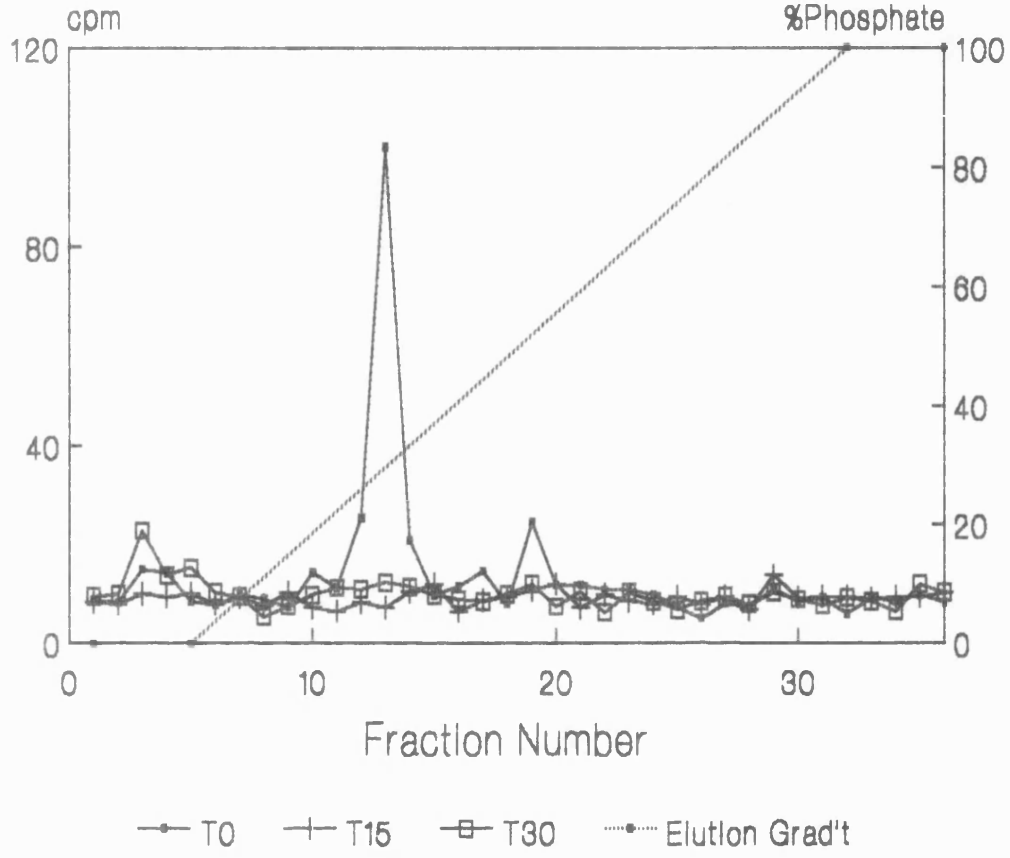


Fig. 3.59. ^{3}H-PIP_2 Turnover Using Electroporation

Strain MC3 (100 ml) was grown to exponential phase and samples (1 ml) were pipetted into electroporation vials. ^{3}H-PIP_2 (0.025 µCi) was added to each vial and the samples were pulsed (2 x 1.5 kV at 1 µFD). These were incubated for 0, 15 and 30 min then harvested by centrifugation. Radioactivity was extracted *via* the method of Bligh and Dyer (1959) and aqueous extracts were analysed by column chromatography. Fractions (2 ml) were collected and mixed with (8 ml) for detection by liquid scintillation counting.

voltage. Electroporation treatment had no effect on the viability of the cells which suggested that either the YEPD medium was an unsuitable buffer for electroporation studies, or the level of pulse used so far was insufficient to breach the cell walls. Investigating the latter possibility first, experiments were performed using increased charges, but no repeatable results were obtained.

Hashimoto *et al.* (1985) and Evans (1991) described the use of SH buffer (10 mM HEPES, 272 mM Sucrose, pH 7.4) in electroporation procedures and an experiment was planned to investigate the viability of cells pulsed at different voltages when resuspended in this solution. The result showed a continuous decrease in viability with increasing pulse voltage and gave further credibility to the theory that YEPD itself was responsible for the lack of success in the previous assays. Subsequent turnover assays with yeast cells electroporated in SH buffer failed to show any ^{3}H-PIP_2 conversion. Although it was possible to record reductions in viability in response to pulse treatment, the results showed very little radioactivity incorporation, no production of novel peaks and unrepeatable elution profiles. There was no evidence for PDE activity in these experiments and alternative methodologies were investigated. It was proposed that cells resuspended in SH buffer might not be able to fully metabolize any incorporated ^{3}H-PIP_2 because they were not in normal growth medium. If the electroporation had been successful, the turnover may not be initiated because of a missing stimulus (e.g. glucose). Resuspension of cells

reduced to 2% viability in glucose or YEPD failed to show any turnover.

The final electroporation experiments using whole cells were performed with the PIP_2/TWEEN 80 buffer as the pulse medium. The buffer was considered to contain a sufficiently high ionic concentration for charge transfer, and it was hoped would allow turnover of the PIP_2 by maintaining an environment similar to the cells' natural interior. The results showed no constant behaviour and the inconsistency of results provoked a fuller study of electroporation in PIP_2 buffer; an attempt was made to try and detect the optimal conditions for ^3H-PIP_2 incorporation. Three experiments were performed using varying pulse characteristics to examine electroporation of yeast cells in this medium.

The only common factor throughout the investigation was a peak at fraction 14. No consistent or repeatable production of IP_3 was monitored and no evidence was provided to suggest actual PIP_2 turnover. Experiments with this methodology were not continued, and techniques to increase the level of ^3H-PIP_2 electroporated in the cells were examined.

3.10.1 The Use of Sphaeroplasts in PIP_2 Turnover Studies

Following the failure of the experiments to incorporate ^3H-PIP_2 into whole cells simply by growing them in a medium containing the tracer (chapter 3.10), a similar experiment

was performed using sphaeroplasts. It was proposed that if sphaeroplasts were incubated in an osmotically buffered medium containing ^{3}H-PIP_2, not only would rebuilding of the cell wall take place (Indge, 1968) the tracer would be more readily incorporated into the membrane for use in turnover studies. No radioactivity was incorporated into the lipid-soluble phase and the uptake appeared unsuccessful. The elution of the aqueous phase also produced a baseline profile and indicated that water-soluble products had not been derived from the incubation. It was not known however if the lack of uptake was due to the absence of a suitable transport system, or whether the formation of sphaeroplasts had rendered the normal uptake mechanisms inoperative. So, an experiment was planned to monitor the uptake of radioactive inositol using sphaeroplasts. A sample of the growth medium (post-harvest) and the aqueous extract showed only the presence of free ^{14}C-inositol. The elution of the deacylated organic phase showed a large peak in the retention position of PI. The inositol tracer had obviously been incorporated into the sphaeroplast membranes, and demonstrated that the normal uptake system, at least for inositol was fully functional. It was concluded that ^{3}H-PIP_2 could not be utilized as a component of a growth medium, as an uptake or transport system did not exist.

Unable to use sphaeroplasts for passive uptake of ^{3}H-PIP_2, a test was performed to see if they could be used in electroporation experiments. The absence of the cell wall may have allowed greater incorporation of the tracer

material - lower charged pulses could be used to penetrate the membranes which was hoped to result in a higher viability. A greater thriving population would therefore remain, and the chance of detecting turnover would be increased. It was only possible to electroporate sphaeroplasts if the osmolarity of the suspension buffer was reduced to 0.8 M sorbitol. This result was repeatable but was unfortunately not usable. The necessity of high charges in an "insulating" buffer resulted in sparks and arcing of electricity across the electroporator as it discharged. This was not considered safe and to protect the machine and operator, further experiments were not performed. It had not been possible to use sphaeroplasts in PIP_2 turnover assays, due to the difficulty in successfully electroporating them. This also meant that it had not been possible to provide evidence for the existence of PDE activity in a yeast cell. Time restraints prevented further investigation but the conversion of exogenously added ^3H-PIP_2 to ^3H-IP_3 using a yeast cell preparation had not been demonstrated.

4.0 DISCUSSION

4.1 Distribution of Activity

The loss of radioactivity from YEPD medium was detectable even in the absence of yeast and subsequent experiments showed that yeast extract and peptone together produced the greatest loss of radioactivity. It was proposed that the yeast extract may have contained enzymes which could degrade inositol and hence release the radiolabel. This was supported by the fact that radioactivity was not lost from an uninoculated flask containing minimal medium, but the presence of proliferating cells caused large losses of radioactivity in both media. It was found that incubating the flasks containing radioactive inositol for 30 min in ice, post-incubation, enhanced the recovery of the tracer and made it possible to reclaim 100% of the added radioactivity. It was thus concluded that ^{3}H was being released as radioactive water vapour during the incubations which was easily recoverable by condensation. The nature of the tritium tracer had indicated that compounds labelled in this manner were not particularly stable (chapter 3.1) and redistribution of the tracer was to be expected. The rapidity in which tritium was lost from inositol was not expected however and doubt was temporarily cast on the ability to perform radiolabelling experiments in yeast. This proved to be unfounded because ^{3}H-inositol was readily incorporated into the cell *via* the active uptake system (Henry, 1982; Nikawa, 1982) and the activity of YEPD on

radioactive compounds did not affect the investigations greatly. Following the initial labelling experiments to detect and isolate inositol phosphates by making the membrane phospholipids radioactive, a majority of experiments involved adding radioactive tracers to previously-prepared yeast cell preparations. These were generally performed in defined buffers for a maximum of 4 h, so loss of radioactivity to the atmosphere was not a major concern. The loss of radioactivity from the medium was not recorded using other chemical tracers over 24 h periods and appeared to be specific to radioactive inositol. This phenomenon had not been reported previously or investigated, but as it only affected a small number of experiments and was easily contained by condensation, experiments were not performed to examine the problem further. The fact that 100% of the radioactivity could be recovered by condensation implied that any tritiated water vapour produced, did not leave the flask. A saturated atmosphere must have existed above the medium and it is possible that the tritium label was constantly exchanged between compounds in the gaseous and liquid phases to maintain an equilibrium. The observation that inositol-derived radioactivity was lost when cells were grown in a medium which did not possess the putative degradation enzymes implied that more than one mechanism was contributing to this activity. It was proposed that the turnover of the incorporated inositol (Angus and Lester, 1972; 1975) to release free ^{3}H-inositol and ^{3}H-GPI into the medium, was partially responsible. The deacylation of radiolabelled membrane PI would involve the

redistribution of tritium between different compounds and it was thought that some of the label may have escaped as ^{3}H-water during these reactions and accounted for the loss of radioactivity. This was not confirmed but the highly hydrophilic nature of inositol may have exacerbated the problem. It was also possible that the radioactive metabolites excreted into the medium were not as stably labelled as the original tracer, which facilitated the transfer of tritium to water molecules. Again, the theory was not confirmed or refuted, nor was it considered important enough to promote further investigation. The incubation of flasks on ice for 30 min after incubation with ^{3}H-inositol was made standard practice and no further problems were encountered.

4.2 Extraction Techniques

The ability to extract metabolites from yeast cells was an important factor in this project. It would not have been possible to monitor turnover of a PI transduction system if the intermediates involved could not be isolated and analysed. During the early labelling experiments it had been noted that even after an extraction technique had been performed on a pellet, a significant amount of radioactivity was still detectable if the cells were resuspended in water and counted in scintillation fluid. An investigation was therefore performed to examine the efficacy of a number of extraction techniques and maximise tracer recovery. The techniques examined (chapter 3.2) showed no significant

differences in mean radioactivity recovery. Cell permeabilization techniques were also examined, but the use of 2-mercaptoethanol or potassium phosphate/isopropanol buffer (Srienc *et al.*, 1983) failed to augment extraction of the radiolabelled metabolites and were not considered exhaustive enough for further use. The use of hot ethanol (Rose, A.H., 1990; pers. comm.) to break or weaken the cells prior to extraction did increase the yield of radioactive metabolites. The use of hot ethanol appeared to function as a single-step extraction procedure as minimal counts were detected in subsequent Bligh and Dyer (1959) extractions and in the resuspended pellet. Shortly after this investigation had been completed, a change to the approach of the project was introduced and this extraction technique was not implemented immediately. It was essential, whatever the extraction method, to be able to separate water and lipid soluble compounds. The method of Bligh and Dyer (1959) was particularly suitable because of the formation of separate organic and aqueous layers and hence this technique was used extensively throughout the project as the standard extraction procedure. This was extremely useful for broken cell extracts and sphaeroplasts. For the exhaustive extraction of lipids, especially from intact cells, the hot ethanol technique was combined with the principles of the Bligh and Dyer (1959) methodology (section 2.15). The Bligh and Dyer (1959) technique was found to be both quick and efficient and facilitated the extraction of both lipid and water soluble moieties. It was not known if the radioactive counts in the pellet that were measured post-extraction

comprised a mixture of lipid-soluble components or just some particular moiety that was resistant to the technique. The thin layer chromatography separations (chapter 3.6) demonstrated that the phosphoinositides were successfully recovered, but it was not known if other inositol-containing membrane components were being removed as efficiently. PI is the major inositol-containing lipid in yeast and contributes 15.8% of the total phospholipid dry weight of the cell (Ratledge and Evans, 1989), but inositol-containing sphingolipids represent 40-60% of the total inositol containing lipids in yeast depending on growth conditions and strain (Lester *et al.*, 1978). This may have represented the radioactivity that was detected in the pellet following ^{3}H-inositol labelling experiments. This would not explain the radioactivity detected after other tracers (e.g. ^{3}H-IP_3, ^{3}H-IP_2) had been used and no evidence was available to substantiate the theory, but if sphingolipids were not extracted as effectively as other lipid compounds, it could explain the remaining radioactivity.

4.3 Radiolabelling and Isolation of Inositol Phosphates

Having demonstrated that at least 90% of added radioactivity could be recovered from inactivated yeast cells, experiments were performed to try and isolate radioactive inositol phosphates. The Bligh and Dyer (1959) technique was used to extract metabolites from cells grown in a medium with radioactive inositol. Analysis by Dowex anion exchange column chromatography showed that water soluble metabolites

were separating into the aqueous phase only (Fig. 3.6) and following sample application and subsequent washing with distilled water, it was possible to demonstrate that further radioactivity was released when an eluting buffer capable of removing $IP_{5/6}$ was applied (Fig. 3.7). It was therefore concluded that the radioactive inositol was being incorporated into the yeast cells and converted into inositol phosphates. Confident that inositol phosphates were being extracted, a stepwise elution system was introduced to separate individual inositol phosphates. The large IP_3 peak originally detected in the elution profile was disproportionately large because the mobile phase used to elute IP_3 also removed IP_2 and IP_1 from the column. Further steps were introduced into the elution profile and it was discovered that the peak seen previously was comprised totally of radioactivity that eluted in the positions of IP_2 and IP_1. No IP_3 was detected. In the initial experiments, cells were incubated in the medium with ^3H-inositol until stationary phase. The premise was that the assay was still in the developmental stage and it was necessary to determine if the extraction could deal with cells in stationary phase, when the cell wall was the most difficult to penetrate. It also allowed for maximal labelling. Although phosphatidylinositol synthase activity remains constant throughout the different growth phases and is unaffected by lipid content (Bae-Lee and Carman, 1990), the PI content of yeast cells is greater in stationary phase than in exponential phase (Homann, 1987). Therefore it was thought that other metabolites related to the PI transduction system

may also be elevated in stationary phase. The extraction failed to show the presence of IP_3, so cells were harvested in mid-exponential phase. As cells enter the stationary phase, metabolic pathways begin to slow down or cease. This was thought to be the reason for non-detection of IP_3. Cells harvested in mid-exponential phase gave the same results as before. No IP_3 was detected but the same levels of radioactivity were detected, indicating that incubation to stationary phase was not essential for maximal labelling. GPI was detected regularly in these assays indicating that some metabolic turnover was occurring, but IP_2 and IP_1 were the only inositol phosphates eluted. This indicated that the PI signal transduction system existed and operated in yeast, and although IP_3 was not detected, it was believed that this was a function of low concentrations of the trisphosphate being metabolized extremely quickly. It was assumed that the peaks were the products of inositol phosphate production, following agonist-mediated hydrolysis of PIP_2. Although a wild type strain was used, it was impossible for radioactive inositol phosphate to have been derived from *de novo* inositol production. Wild type cells convert glucose-6-phosphate to inositol *via* inositol-1-phosphate (Culbertson *et al.*, 1976) and this IP_1 can be extracted from the cytosol. IP_1 would not be detectable though as it would not be radiolabelled. The exogenous radioactive inositol is taken into the membrane and incorporated into phospholipid PI. Radioactive IP_1 could only be produced as a result of the hydrolysis of PI itself or by the dephosphorylation of IP_3 to IP_2 to IP_1. It was discovered however that the lower

inositol phosphate peaks detected previously were not comprised of *bona fide* inositol phosphates, but were radiolytic decomposition products, (derived from the ^{3}H-inositol) which co-eluted with the lower inositol phosphates. This was confirmed by eluting a sample of the tracer from a Dowex resin column. The same peaks were detected in the "pure" tracer sample and it appeared as if these contaminant peaks were solely responsible for the IP_2 and IP_1 peaks detected previously. It could be concluded that the elution system functioned properly as the two peaks were successfully separated. Doubt was then cast on the existence of the signalling pathway because no inositol phosphates had been detected. Further experiments were curtailed until a different inositol tracer could be obtained in which the decomposition could be retarded or halted. It was also planned to use inositol-requiring strains to augment the level of ^{3}H-inositol incorporated. Inositol auxotrophs of *Sacch. cerevisiae* were described by Culbertson and Henry (1975). Inositol-1-phosphate synthase from yeast has been purified (Donahue and Henry, 1981) and found to be a tetramer of approximately 240,000 Da, consisting of identical subunits of approximately 62,000 Da. The *INO1* locus is the structural locus for the single subunit of Ins(1)P_1 synthase and mutations at this locus eliminate the synthase activity (Donahue and Henry, 1981; Culbertson *et al.*, 1976). Starvation of *ino*1 mutants for inositol leads to an immediate decline in the rate of synthesis of PI (Henry *et al.*, 1977; Becker and Lester, 1977) but there is no coupled decrease in

phosphatidylcholine synthesis. Inositol auxotrophs are totally dependent on external inositol, and using such an inositol-requiring mutant in labelling experiments it was hoped to improve both the rate and level of incorporation of ^{3}H-inositol. Increased levels of ^{3}H-inositol in the medium were also considered as a method of raising the radioactivity of any PI turnover metabolites. There was no evidence for the transduction system in yeast.

4.4 Detection of Inositol Phosphates

Genuine inositol phosphates were detected and separated when a novel approach to labelling the cells was used. The introduction of inositol-requiring mutants and a period of incubation in a medium without inositol appeared to promote rapid uptake of ^{3}H-inositol when added subsequently and turnover of the labelled phospholipids. It also avoided long term incubations which promoted loss of ^{3}H-inositol. Using the new methodology (section 2.11) it was possible to detect radiolabelled GPI, IP_1 and IP_2. The GPI was just a function of the deacylation of PI and had been previously detected, but the inositol phosphate peaks had not been seen before. Elution of the tracer material from an anion exchange column showed no contaminant peaks and the result was considered to demonstrate lower inositol phosphate production. Inositol bisphosphate was detected when the assay was extended to 30 and 60 min (Fig. 3.10) and showed an increase over this time. Kaibuchi *et al.* (1986) stated that although they had detected both IP_1 and IP_3, they had not detected IP_2.

Considering that IP_2 is produced only as a result of IP_3 dephosphorylation (or PIP hydrolysis, Downes *et al.*, 1989) and IP_1 is produced from the dephosphorylation of IP_2 , this seemed to be a very unusual observation. Kaibuchi *et al.* (1986) also used inositol-requiring mutants, so production of IP_1 *via* Ins(1)P_1 synthase was impossible and had to be derived from IP_2 or the hydrolysis of membrane phosphoinositides. The elution technique of Kaibuchi *et al.* (1986) would have separated IP_3, IP_2 and IP_1 into their correct fractions, so the result did not appear to be mis-identification. The results of Kaibuchi *et al.* (1986) implied that the inositol-1-phosphate detected was not derived from the dephosphorylation of IP_2, but from the hydrolysis of PI. This would explain the complete absence of IP_2, as an inositol phosphate dephosphorylation pathway did not appear to be functioning. It is not clear why PI and PIP_2 were hydrolysed by phospholipase C activity as a result of glucose-stimulation, and PIP was not. The conclusion must be drawn from the results of Kaibuchi *et al.* (1986) that an inositol phosphate pathway does not exist or operate in yeast. Over the period of the incubation (60 min) there was no reported decrease of either IP_3 or IP_1, and no report of IP_2 production.

The results illustrated in chapter 3.5 show the isolation of IP_2 and IP_1. The level of IP_2 was seen to decrease with time and IP_1 and GPI increased. These results contradict those described above in two ways:-

1] Throughout the investigations, IP_2 was isolated regularly but an IP_3 peak was never detected.

2] The levels of the inositol phosphate fluctuated with time, indicating the presence of a phosphatase - based degradation system

This was the first positive evidence for the existence of a transduction system in yeast. Although IP_3 had not been detected, the appearance of inositol phosphates in the elution profile and the degradation of IP_2 to IP_1 showed that some of the pathway intermediates were present. Despite a ten-fold increase in the concentration of exogenously added ^3H-inositol in the medium, and the inclusion of unlabelled carrier inositol, it was not possible to demonstrate IP_3 production. It was concluded that the trisphosphate must have been produced in very small concentrations and metabolized rapidly. This did not seem to agree with the observed results - a small IP_1 peak had been detected after 10 min, but IP_2 had only been detected after 30 min. If IP_3 had been converted rapidly, then IP_2 should have been produced before IP_1. Although the level of IP_2 decreased after its initial detection, the results implied that the normal mammalian-like turnover system was not operating in yeast. The implication was that the IP_2 was produced as a result of the hydrolysis of PIP. Following hydrolysis, IP_2 was released into the cytosol and subsequently degraded. This also meant that the IP_1 peak detected originally was probably produced by the hydrolysis

of PI. It was considered that IP_2 was the second messenger in yeast as opposed to IP_3, but no evidence was available to support this.

Experiments to induce the receptor-mediated hydrolysis of PIP_2 to produce IP_3 by including a glucose or glucose/nutrient stimulus were unsuccessful. If the previous results were due to the splitting of the inositol phospholipids and only IP_2 and IP_1 had been detected, then the reason IP_3 had not been observed was because the right stimulus to initiate the hydrolysis of PIP_2 was not present. The premise was that PI and PIP may be regularly hydrolysed in the normal metabolism of the yeast cell, but the hydrolysis of PIP_2 to release the second messenger required specific conditions and stimuli. No IP_3 was detected in any of the glucose-stimulus experiments and the results obtained were again contrary to those of Kaibuchi *et al.* (1986). Peaks eluting in the positions of GPI, IP_1 and IP_2 were detected regularly but the inclusion of glucose "stimulus" had had no effect on the results. The effect of glucose is discussed further in chapter 4.6.

Although IP_3 had not been observed, it had been possible to isolate and separate some of the lower inositol phosphates (GPI, IP_1 and IP_2) from yeast. Hence some kind of inositol-based turnover was occurring although the source of the inositol phosphates was not entirely clear. This implied that the IP_2 and possibly some of the IP_1 were derived from the hydrolysis of PIP and PI. Degradation of IP_2 was

monitored however, with the concomitant increase in IP_1 and this indicated that the inositol phosphate degradation pathway possibly existed in yeast. Some evidence was therefore available to suggest the PI transduction system was present, but the crucial PIP_2 to IP_3 conversion was not shown.

4.5 Background Evidence for the Transduction System

Using simple labelling techniques, it was possible to monitor the incorporation of radioactive inositol into proliferating yeast cells. Evidence was required to show that the tracer was being effectively taken up from the medium and distributed amongst the inositol phospholipids. It was thought that the inability to demonstrate the presence of IP_3 was a result of inadequate labelling of the precursor PIP_2. Having shown that the uptake of ^{3}H-inositol from the medium followed the same time course as the growth curve, it was concluded that if cells in early exponential phase were resuspended in fresh medium containing ^{3}H-inositol, then maximum labelling could be achieved in a period of 5-6 hours (see Fig. 3.13). It was not therefore essential to grow cells to stationary phase in ^{3}H-inositol to obtain maximal labelling. Longer incubations provided higher levels of radioactive counts simply as a result of higher cell densities. A subsequent experiment (Fig. 3.14) appeared to show 2 peaks in the radioactivity per cell, possibly implying that separate inositol pools were equilibrating during incorporation of the tracer. Many

labelling studies in mammalian cells suggested that the inositol lipid, which is hydrolysed during transmembrane signalling in response to receptor activation, might be drawn from a metabolically discrete and relatively small, hormone-sensitive pool that turns over more rapidly than the bulk of membrane inositol lipid. It was not known if the 2 peaks represented incorporation into the membrane fraction, cytosol or both, but the two-stage cell labelling curve indicated that inositol uptake involved multiple inositol-sink pools. Dual labelling experiments in mammalian cells (Maccallum *et al.*, 1989) showed that hormone stimulated inositol lipid hydrolysis can call, either directly or indirectly, upon the majority of the inositol lipid complement of the stimulated cell. If separate inositol pools are present, they did not greatly affect the availability of inositol in the mammalian cells investigated. It is not known how relevant this information was to yeast cells, and the matter was not investigated further.

Using TLC demonstrated that incorporated radioactive inositol was distributed to PI, PIP and PIP_2. The lipids were successfully labelled but were not detected in a ratio that had been recorded previously. This may be due to variations in strain, growth phase or the method of assay. The levels of radioactivity incorporated into PIP_2 were generally very low, and this might be a major factor in the inability to detect IP_3. The very low levels of labelled PIP_2 promoted investigation of the 2 separate enzymatic

steps i.e. the breakdown of exogenously added ^{3}H-IP_3 using cellular material and the conversion of exogenously added ^{3}H-PIP_2. The very low levels of PI in the yeast membrane appeared to make labelling PIP_2 to a sufficiently high level very difficult and hence further labelling experiments were not performed in this manner.

Yeast was shown to actively take up inositol tracer from the growth medium, and distribute the radioactivity to the phosphoinositides. This provided excellent background evidence for the transduction system in yeast and promoted investigations into the assay of IP_3 phosphatase and phospholipase C activity.

4.6 Glucose as a Stimulus for PI Signal Transduction

Turk *et al.* (1986) reported that the accumulation of inositol trisphosphates in pancreatic islets could be induced by glucose, and this premise was used by Kaibuchi *et al.* (1986) in their investigations into inositol phospholipid turnover in yeast. Until very recently, this was the only report of the detection of IP_3 in *Sacch. cerevisiae* and therefore experiments were performed to attempt to reproduce the findings. The investigation of the techniques used by Kaibuchi *et al.* (1986) is described in chapter 3.7, in which little evidence was found to support the recorded observations.

Although the initial experiments were contaminated by the presence of radiolytic decomposition products of the tracer inositol, a large IP_1 peak was detected which could not be accounted for by the co-eluting radioactivity. This showed glucose-induced ^3H-IP_1 production, but there was no evidence of IP_3 production. This result was also demonstrated by Hawkins *et al.* (1992b) who also repeated the methodology of Kaibuchi *et al.* (1986) to investigate further the response of yeast to glucose stimulation. This suggested that IP_1 is the only water-soluble inositol phosphate product of glucose induced turnover.

To eliminate the possibility of contaminant peaks affecting the elution profiles, a number of experiments were performed using ^{14}C-inositol. Using the same methods, peaks corresponding to GPI, IP_1 and IP_2 were detected. This gave further support to the presence of an inositol phospholipid transduction system in yeast. An inositol trisphosphate peak was still not detected, but this might be a result of the rapid degradation of the second messenger. Detection of IP_2 and IP_1 implied that an inositol phosphate degradation pathway was present in yeast to dephosphorylate IP_3. These results also reinforced the observations described in chapter 3.5, when IP_2 and IP_1 were isolated and separated. The detection of IP_2 was not described by Hawkins *et al.* (1992b) who stated that the majority of water-soluble ^3H-radioactivity in yeast was associated with GPI and IP_1 and there was nothing corresponding to Ins(1,4)P_2 or Ins(1,4,5)P_3. It is not known if the reason for this

variation in results was due to the use of different strains or slightly different methodologies, but the main feature was the common failure to detect IP_3. The theory that IP_3 remained undetectable as a result of inadequate radioactive labelling did not obtain. Increasing the concentration of radioactive inositol to a concentration of 10 or 20 $\mu Ci\ ml^{-1}$ did not provide any extra information concerning the release of water-soluble metabolites to the cytosol and implied that any turnover recorded was unaffected by the presence of glucose (Fig. 3.23).

Further information was not obtained until the tertiary supernatant (see chapter 3.7) was removed for independent analysis. Perhaps (Fig. 3.24) the inclusion of the tertiary supernatant greatly affected the results and implied that very little inositol phospholipid turnover was manifested within the cell. The conclusion made was that GPI production increased in response to glucose stimulation with an accompanying decrease in the level of free inositol. Hawkins *et al.* (1992b) also described a large increase in GPI, but they also described the isolation of a highly polar metabolite in the water-soluble extracts with the chromatographic characteristics of inositol hexakisphosphate, together with trace amounts of radioactivity in compounds which chromatographed very closely with Ins(1,3,4,5,6)P_5 suggesting the presence of IP_5 isomers. Neither the results of Kaibuchi *et al.* (1986) nor those described in chapter 3.7 detected any of the higher phosphorylated inositol phosphates, but the contrasting

observations may, again, be due to variations in methods and strains used. The presence of IP_5 and IP_6 in yeast would suggest that a very well developed inositol phospholipid signalling system existed in yeast but this was not substantiated by the corresponding isolation of IP_3 or IP_2 in the case of Hawkins *et al.* (1992b) and Kaibuchi *et al.* (1986). It seems more likely that lower inositol phosphates are produced more readily and detected easily, particularly IP_3 and its dephosphorylation products, in a cell in which the transduction system was not known to be established. The absence of published information on the detection of IP_4 in yeast also argues against the presence of a fully developed system in yeast. Although some IP_3 kinase activity can be demonstrated (chapter 3.8), IP_4 has never been isolated from yeast and considering the important role of this intermediate (see Introduction) in mammalian cells, this does not support a mammalian-like transduction system in yeast. If IP_3 and IP_4 cannot be isolated from yeast after a turnover stimulus, it suggests that a system different from that in mammalian cells exists, and it seems unlikely that yeasts undergo the complex reaction pathways to produce highly phosphorylated inositol phosphates which have poorly-defined functions.

The independent elutions of tertiary supernatants, essentially the reaction buffer, indicated that the main response to glucose stimulation was extracellular. It was observed initially that, post stimulation a novel "IP_1" peak could be detected in the medium (Fig. 3.27). Following

the discovery that the incubation period for the cells to reach stationary phase had to be extended to 48 h (Fig. 3.28), it was then possible to detect peaks corresponding to inositol, GPI, IP_1 and IP_2 in the medium post stimulation (Fig. 3.29-3.32). It was proposed that these peaks did not correspond to genuine inositol phosphates and were not therefore the products of phospholipase C activity. Although water-soluble, it was not expected to find inositol phosphates in the reaction medium. It was suggested (Downes, C.P., 1992; pers. comm.) that these compounds were the deacylation products of the phosphoinositides formed by phospholipase A or B activity. Hawkins *et al.* (1992b) made similar observations and performed "head-group" analysis on the compounds. Their observations confirmed the identities of the novel extracellular peaks as GPIP and $GPIP_2$, glycerophosphatidyl inositol phosphates. Using glucose as a stimulus, it appeared that GPI and GPIP levels increased irrespective of the glucose, but $GPIP_2$ levels only increased as a result of stimulation. No increase in $GPIP_2$ was recorded in the absence of glucose. Hawkins *et al.* (1992b) reported that GPIP and $GPIP_2$ were the major water-soluble polyphosphoinositol metabolites produced in response to glucose, and there was no evidence for the phospholipase C-dependent production of IP_3. It was concluded that glucose acted as a stimulus for turnover of the inositol phospholipids but not for the activation of phospholipase C. The results of Kaibuchi *et al.* (1986) are difficult to explain but may represent differences in chromatographic conditions or procedures. No evidence was available to

support their observations and IP_3 remained undetectable. The observation by Uno *et al.* (1988) that glucose affects PIP_2 levels remains valid, but this turnover cannot be associated with the products IP_3 and DAG.

It was not possible to detect the deacylation product of PIP_3 (chapter 3.7; Hawkins *et al.* 1992b). As PI(3)P was found to comprise 60 % of the total PIP content (Hawkins *et al.* (1992b), it was a strong indicator that PIP_3 may have been present. PI(3)P is produced following the hormone-stimulated phosphorylation of PI(4,5)P_2 to PI(3,4,5)P_3 and its subsequent dephosphorylation to PI(3,4)P_2 and PI(3)P (Stephens *et al.*, 1991). Only weak chromatographic evidence was found for PI(3,4)P_2 however and it is not known if yeast cells use this pathway or if suitable conditions were used to detect it. Glucose, in this case may not be an appropriate stimulus. Hawkins *et al.* (1992b) also noted that the proportion of PI(3)P to PI(4)P remained constant both before and during glucose-stimulation, but the extracellular GPIP was entirely composed of GPI(4)P. This meant that either the lipid substrates were highly compartmentalized or the hydrolytic activities were very phosphate-group specific.

Although cell-cycle arrested yeast cells are able to remain viable for long periods (Lillie and Pringle, 1980), Granot and Snyder (1991) reported that stationary cells incubated in the presence of only glucose lost viability rapidly, at a similar rate or faster than those in synthetic growth medium

(glucose plus nitrogen source). This loss of viability occurred for both haploid and diploid cells. If the cells were provided with additional nutrients, morphological and physiological changes characteristic of mitotically growing cells were induced. These changes were found to be induced specifically by D-glucose and were independent of the adenylate cyclase pathway. Granot and Snyder (1991) suggested that glucose triggers events in the induction of a new mitotic cell cycle and that these events were either prior to the adenylate cyclase pathway or used an alternate pathway. It was proposed that as glucose induced an influx of calcium ions in yeast (Eilam and Othman, 1990), and the phosphatidylinositol turnover system is associated with such an influx (see Introduction) and other cellular signals in mammalian cells, then the same signalling system could be involved in the induction of early cellular events. Although supported by Kaibuchi *et al.* (1986) and Uno *et al.* (1988), the results in chapter 3.5, 3.7 and Hawkins *et al.* (1992b) imply that glucose is not an appropriate agonist and could not initiate this signal transduction. There was no evidence of PI pathway second messenger production in response to glucose and no indication that the system actually existed in yeast. According to the most recent data, it seems unlikely that the cell cycle induction was mediated through the PI transduction system as a response to glucose.

These observations are supported by the recent publication of Schomerus and Küntzel (1992) who claim not only the detection of IP_3 in yeast, but also that the two major

nutrient sources glucose and ammonium elicited specific responses in starved yeast. Glucose induced a transient cAMP signal and ammonium stimulated a sustained increase of DAG and IP_3, but not *vice versa*. This elevated nitrogen-induced IP_3 level remained constant for up to 60 min (similar to the glucose-induced observation of Kaibuchi *et al.*, 1986), but the elevated IP_3 and IP_1 levels reported as a result of glucose-induced turnover were not recorded by Schomerus and Küntzel (1992). The response of IP_3 was reduced by dissecting the RAS-activating Cdc25 protein, and was completely abolished by the *cdc*25-1 mutation even at the permissive temperature. The effect depended on a functional Cdc25 protein which was required to activate RAS proteins by catalysing the exchange of GDP by GTP on Ras. This suggested that the Cdc25 protein controlled a novel signalling pathway - the nitrogen-induced accumulation of IP_3 and DAG. It may represent a highly specific system as glucose does not induce PIP_2 turnover and nitrogen does not appear to induce cAMP. The data of Schomerus and Küntzel (1992) are difficult to evaluate fully, as no other intermediates of the pathway are discussed. The prolonged elevation of IP_3, reminiscent of the result of Kaibuchi *et al.* (1986) implied that no phosphatase or kinase activity is present to deactivate the potential second messenger. This seems unlikely, and the lack of reference to phospholipid hydrolysis and deacylation make it uncertain as to what is actually being assayed. It was not shown if the assay method used could differentiate between IP_3 and $GPIP_2$ which elute very closely from anion exchange columns, or can even co-elute depending on the

regime used. It is very doubtful that Schomerus and Küntzel (1992) actually measured only IP_3 in their investigations as they failed to separate the cells from the reaction buffer prior to assay. It is probable that the compounds measured included extracellular $GPIP_2$ which would explain the extended elevated concentration. The separation of cells and medium was shown to be vital (chapter 3.7) for the analysis of inositol phospholipid turnover and further work is essential for this publication to be considered useful. The only salient point about their results is that glucose did not affect turnover, but nitrogen apparently did. This is further evidence against the initial observations of Kaibuchi *et al.* (1986).

Following the work of Ohya *et al.* (1984) which showed that Ca^{2+} ions and calmodulin played important roles in the yeast cell division cycle at the stage of bud growth and nuclear division, a method was developed for measuring cytosolic Ca^{2+} and its time dependent changes in yeast by using the luminescent protein aequorin as a Ca^{2+}-specific indicator (Nakajima-Shimada *et al.*, 1991). Using this method it was observed that glucose added to glucose-starved G_o/G_1 cells stimulated an increase in extracellular Ca^{2+}-dependent luminescence with maximal intensity occurring 2 min after addition. Kaibuchi *et al.* (1986) showed Ca^{2+} efflux occurred in yeast concomitant with the $^{32}P_i$ incorporation into inositol phospholipids and these two events followed similar kinetics, but changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were not demonstrated upon glucose

addition. Similar results were obtained when fructose or mannose was added to the glucose starved cells, but non-metabolizable sugar analogues such as 2-deoxyglucose and 6-deoxyglucose did not induce this effect. This agrees with the results of Kaibuchi *et al.* (1986). Specific roles for Ca^{2+} have been found in *Sacch. cerevisiae* (Iida *et al.*, 1990), particularly during mating and the cell cycle. The addition of glucose caused a transient rise in the $[Ca^{2+}]_i$, but as the mechanism of the response was unknown, further experiments were performed. The response was shown not to be associated with an increase in cAMP levels, was not dependent on *de novo* protein synthesis and was potentially energy dependent (Nakajima-Shimada *et al.*, 1991). The results suggest the turnover of PIP_2 to produce the calcium-releasing second messenger IP_3 in response to glucose, and support the observations of Kaibuchi *et al.* (1986).

The reaction of starved yeast cells to a glucose stimulus has yet to be universally agreed. The majority of evidence suggests phosphoinositide turnover is not mediated by a glucose stimulus to produce IP_3 and DAG. Instead, glucose appears to promote a transient increase in intracellular calcium and can increase phospholipase A or B activity to produce deacylated extracellular GPI, GPIP and $GPIP_2$. Perhaps IP_3 production is stimulated by nitrogen, but whether the cAMP pathway is involved with the addition of glucose is not clear. A calcium cascade could be initiated without the production of IP_3, and may be part of a completely separate pathway. Also, it is possible that the

turnover of PIP_2, and the release of intracellular calcium do behave in mammalian-like responses, if the correct agonist is administered. The results in chapter 3.7 showed an increase in GPI, GPIP and $GPIP_2$ in response to a glucose stimulus. This presumably entailed a decrease in concentration of the precursor phosphoinositides. It is not known if this purported loss of phospholipid is important and involves some reconfiguration of the membrane itself or is just a by-product of a more important reaction or signalling system. Perhaps agonist-stimulated release of glycerophosphoinositides is a method of communication between cells, and the released products may themselves have some agonist properties. It is equally possible that the responses seen were simply induced by nutritional or growth influences mediated by glucose, but Hawkins *et al.* (1992b) do not believe this to be the case. In their results, the very different proportionate changes from control levels in the various inositol metabolites, the specific effects of both temperature and growth phase on GPIP/$GPIP_2$ formation and the nature of the responses in *cdc* mutants to glucose pointed to separate regulation of polyphosphoinositide metabolism.

4.7 The Metabolism of Exogenously Added ^3H-IP_3

Despite initial failures, it was eventually possible to demonstrate the breakdown of exogenously added ^3H-IP_3 in a yeast cell preparation. Early experiments were thought to have failed because essential co-factors were not present or

the incubation buffer was inhibitory. Believing the phosphatase activity to be present, improved buffers were introduced but it was still not possible to demonstrate IP_3 degradation. To ensure that cell breakage was occurring, homogenisation in a Braun shaker was used instead of sonication to disrupt cells but evidence for the breakdown of ^{3}H-IP_3 was only obtained using the method of Hanson (1991). Primary results indicated that the level of IP_2 was increasing with time, but no reduction in the level of IP_3 was recorded. Prompted by this observation, extended assays were performed in which the IP_3 level decreased with time. This was the first evidence for the presence of an IP_3-degrading enzyme, but it was also noted that IP_2 and IP_1 were also degraded in a similar fashion. No concomitant increase in any of the lower inositol phosphates was recorded with a decrease in IP_3 levels. This suggested that an inositol phosphate phosphatase pathway was not responsible for the reduction in peak height. The slow rates of reaction and the lack of production of lower inositol phosphates implied that non-specific or opportunistically acting enzymes were responsible for the results. This was further compounded by the inability of chloride ions to inhibit the degradation, and the failure of a Hanson (1991) preparation to breakdown exogenously added ^{14}C-IP_1. The unavailability of tracer IP_2 prevented assays being performed on this substrate, which may have provided further information about the enzymes under investigation. A degradation of IP_2 without coincident IP_1 production would have supported the results already seen, but if IP_2 was

rapidly broken down with a concurrent increase in monophosphate concentration, then it may have suggested that an IP_2 phosphatase was present, which had a high specific activity. The inability to extract IP_3 from a radiolabelled yeast extract, and the regular detection of IP_2 suggested that this intermediate plays a more important role and is possibly used by yeast as a second messenger instead of the trisphosphate. The results did not support this hypothesis and it was concluded that the profiles seen could not be attributed to a mammalian-like inositol phosphate degradation pathway. All the preparation procedures had involved harsh treatment of the cells prior to assay and it was possible that the use of sonication or homogenisation in a Braun shaker had denatured the enzymes. A more gentle approach to cell preparation was investigated.

The use of cationic silica micro-beads to produce membrane preparations is a gentle technique, but no relevant information about phosphatase activity was gained. The micro-beads sequestered exogenously added radioactivity and could not be used to demonstrate any genuine enzymatic activity. However, the membrane preparation experiments prompted the use of broken sphaeroplasts and this gave the main evidence for a phosphatase degradation pathway in yeast. Initial experiments were analysed by elution from 10 cm Dowex resin columns attached to the HPLC and demonstrated an increase in IP_2 and IP_1 concentration as IP_3 was degraded. The results were enhanced using a 10 µm SAX HPLC column. This showed that the increase in IP_1 occurred after

a transient increase in IP_2, as IP_3 was degraded. An increase in free inositol (the end product of the degradation pathway) was also shown. Further experiments demonstrated the degradation of exogenously added ^{3}H-IP_2 and ^{14}C-IP_1. This was accompanied by coincident increases in lower inositol phosphates and inositol. Thus, the inositol phosphate phosphatase degradation pathway was established in yeast and further evidence was provided for the existence of the PI transduction system. It had been noted however that the rates of reaction in the turnover studies were relatively slow, some experiments took up to 4 h to show the production and subsequent degradation of IP_1 in IP_3 turnover assays. This was contrary to the notion of rapid turnover of the inositol phosphates had prevented detection of IP_3 in previous radiolabelling assays. It was also not possible to inhibit the degradation of the radioactive substrates with Li^+. Although the distribution of IP_3 phosphatase activity between membrane and cytosol was not known, the use of broken sphaeroplasts ensured that all the cellular material was included in the assay and the slow reactions could not be due to missing cell components. Faster reactions were recorded in the IP_2 phosphatase assays suggesting that IP_2 may be more important than IP_3. The cells appeared to remove exogenously added ^{3}H-IP_2 much more quickly than ^{3}H-IP_3 which implied that IP_2 had a second messenger function and was produced by the receptor-mediated hydrolysis of PIP. The inability to halt degradation with Li^+ may have been because of inadequate concentrations. The levels of Li^+ in the reactions were derived from mammalian cell experiments and

these may not have been sufficient to inhibit yeast reactions. Perhaps the degradative pathway is present, but differs from that in higher eukaryotes.

It is probable that the attempts to assign specific activities to the inositol phosphate phosphatases were experimentally flawed. It is likely that the concentrations of tracer used in these experiments were well below the saturation levels for the putative enzymes. The activity detected would therefore be proportional to the amount of substrate supplied and the reactions may not have proceeded at Vmax. The substrate would have been limiting and hence the result would not be a true reflection of the enzyme's activity. As neither equimolar amounts of the tracers were used nor the same number of radioactive counts, the results are not strictly comparable. The extremely low turnover rates recorded implied that the degradation was not mediated by enzyme activity. Further information could however be gained if experiments were performed using a range of tracer concentrations, then turnover rates could be associated with substrate levels. It may then be possible to draw conclusions about the specific activities.

Three independent degradations were monitored, possibly resulting from phosphatase activities, but the rates of reaction were not as expected. Further evidence was provided for the existence of an inositol phosphate degradation pathway in the yeast *Sacch. cerevisiae*, but it would seem

unlikely that the IP_1 phosphatase activity would be more than 1500 times faster than the IP_3 phosphatase activity if the system functioned in the same way as in mammalian cells where the primary role is to halt the activity of IP_3. An extremely fast activity for IP_3 phosphatase would also help to explain the inability to detect IP_3 in a yeast cell extract. The results did not support this premise and it could be concluded that IP_3 was either produced in very small quantities or not at all. The result obtained indicated that the phosphatase enzymes present were more specific for IP_2 and IP_1 degradation, perhaps implying that these inositol phosphates were more important intermediates in yeast metabolism than IP_3. It could also be concluded however that the IP_3 phosphatase activity was distributed between both the membrane and cytosolic fractions. The activity recorded with the broken sphaeroplasts (1.39×10^{-12} mmol mg^{-1} ml^{-1} min^{-1}) was faster than that observed using just the cytosolic fraction (8.8×10^{-14} mmol mg^{-1} ml^{-1} min^{-1}). No other information was available to compare these results, but factors including strain, growth conditions and methods of analysis may effect the activities of the enzymes making it difficult to draw general conclusions.

IP_3 kinase activity was monitored to investigate whether, in yeast, IP_3 was preferentially deactivated by conversion to IP_4. Although an IP_3 kinase was definitely present, it did not have the turnover characteristics predicted for the removal of a second messenger. In the buffer used both IP_3

kinase and IP_3 phosphatase were acting simultaneously, and IP_3 phosphatase had the higher specific activity. Although this provided more evidence for the existence of the PI transduction system in yeast, by isolating a new enzyme and intermediate, it did not explain the inability to detect IP_3. The evidence suggested that ^3H-IP_3 was not degraded at a particularly fast rate, either by a kinase or a phosphatase. It must be concluded that IP_3 was not produced or was undetectable due to its presence in very low concentrations.

IP_2 kinase activity either does not exist or operate in yeast. This agreed with the observations made in mammalian cells (Downes *et al.*, 1989) but possibly contradicts the theory that IP_2 may act as a second messenger. The discovery of IP_3 phosphatase and kinase activities suggested that, as in mammalian cells, IP_3 plays a more important role than IP_2. Further work is required to substantiate this and investigate the specificity of the kinase. Perhaps the results obtained *in vitro* bore no resemblance to activities *in vivo* and the actual turnover of inositol phosphates is actually much faster. The elucidation of the phosphatase degradation pathway and the existence of IP_3 kinase in yeast have shown parts of the signalling system are common to both simple and higher eukaryote cells. It is not known however if these pathways have common functions or respond in the same way.

4.8 The Assay of Phospholipase C

Throughout the investigation, it was not possible to demonstrate the conversion of cellular PIP_2 to IP_3 or the hydrolysis of exogenously added ^3H-PIP_2 using a variety of techniques and cell preparations. This implied that phospholipase C does not exist or operate in yeast and the PI signal transduction system does not function as in mammalian cells. Kaibuchi *et al.* (1986) and Schomerus and KÜntzel (1992) reported that PIP_2 turnover could be stimulated by glucose and nitrogen respectively with the subsequent production of IP_3, but these results may be attributable to the production of $GPIP_2$. No conclusive evidence exists for the receptor mediated hydrolysis of PIP_2 to DAG and IP_3 in yeast.

Labelling studies showed that PIP_2 was definitely present in the plasma membrane, and glucose-stimulation of starved, cells showed that the deacylation by-product of PIP_2 could be produced as a result of phospholipase A activity. Evolution may have removed the triphosphoinositide if it was not essential, although the possibility of a vestigial existence must not be ignored. Conversely, inositol phospholipids in yeast may be simply for the maintenance of structural integrity of the membrane, especially if the enzymes required to metabolize PIP_2 are not present. Yeast may degrade PIP instead of PIP_2 and utilize IP_2 as a second messenger. This would help to explain the detection of IP_2

and IP_1, and the inability to observe IP_3 production. It is possible that the signalling system exists and operates in yeast, but the correct conditions and stimulus were not applied, or the inability to detect PIP_2-derived IP_3 was due to insufficient radioactive labelling or damage to the enzyme or phospholipid during the assay. The absence of evidence to show receptor-mediated hydrolysis of PIP_2 to the second messengers is a major hindrance to the notion of a transduction system in yeast. Without this evidence, a mammalian-like pathway can not be concluded to operate in yeast. Further experiments, comparing the behaviour of the putative pathway under varying conditions were not performed due to insufficient evidence.

In summary, the uptake of ^{3}H-inositol and distribution to the inositol phospholipids provided good background evidence for the existence of the pathway in yeast, and the regular detection of peaks that eluted from an anion exchange column with the retention properties of IP_2, IP_1 and GPI indicated that some kind of turnover was taking place. The inability to detect IP_3 was a complicating factor, but it was initially thought that rapid metabolism and low concentrations were responsible. A wide range of techniques and assays were focussed on the production/detection/breakdown of IP_3 in yeast because of the ubiquitous nature of the trisphosphate in higher cells. It was essential to eliminate all aspects of experimental error and ambiguity before the presence or absence of this intermediate could be declared. Evidence for the absence of

IP_3 included its undetectability and the inability of yeast cell preparations to convert exogenous PIP_2 to IP_3. Conversely, enzymes capable of dephosphorylating IP_3 to IP_2 and further phosphorylating the trisphosphate to IP_4 have been detected and assayed. In conclusion, the results would appear to suggest the presence of a transduction pathway, but one in which IP_3 does not necessarily play a major role. The signal transduction system established in mammalian cells which is based on the activation of phospholipase C, leading to the formation of DAG and IP_3 does not appear to operate in the yeast *Sacch. cerevisiae*. Hawkins *et al.* (1992b) proposed that this was not particularly surprising since this system is designed in higher cells to allow cell-cell communication (by coupling cell-surface receptors to intracellular metabolism) and yeast is a unicellular organism where, as far as is known, the only intercellular communication is *via* the mating pheromone system.

5.0 References

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